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(57) Abstract

The present invention provides methods for producing and purifying proteins comprising TGF-β3 precursor, pro region of the TGF-β3 precursor and mature TGF-β3. Moreover, this invention also provides methods for producing and purifying novel homodimeric proteins with enhanced production and heightened therapeutic utility which comprises mutations, signal peptide deletions, and protease cleavage site substitutions.

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TISSUE-DERIVED TUMOR GROWTH INHIBITORS, METHODS FOR PREPARATION AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 353,410, filed May 17, 1989, which is a continuation-in-part of U.S. Serial No. 183,224, filed April 20, 1988, which is a continuation in part of U.S. Serial No. 111,022, filed October 20, 1987, which is a continuation-in-part of U.S. Serial No. 922,121, filed October 20, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 847,931, filed April 7, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 725,003, filed April 19, 1985, now abandoned, the contents of each are hereby incorporated by reference int the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known t those skilled therein as of the date of the invention described and claimed herein.

Transforming growth factor β (TGF-β) is part of a family of multifunctional proteins which appear to modulate, alone or in combination with other molecules, cell proliferation and differentiation. Reportedly, TGF-β, which comprises both a mature, a pro and a precursor form, includes several isoforms (i.e. TGF-β1, -β2. -β3, -β4, and -β5) coded for by different

g n s. The TGF-β2 and TGF-β3 cDNA have been found from several mammalian s urces (14). TGF-β4 cDNA has nly been is lated from chicken cells (17). Mature TGF-β1, -β2, -β3 cDNAs share an amino acid sequence identity of about 75-80% whereas the TGF-β1, -β2, -β3 precursor exhibit only 25-35% identity. Surprisingly, despite the high degree of homology among the TGF-β's, it appears that these proteins have distinct differences in potencies (14).

Mature TGF-β has been isolated from various species. Murine, bovine, human, and porcine TGF-β have been isolated and show very little difference in amino acid composition (5, 8, 11, 14 24).

The cDNA sequence of mature TGF-β, its expression in both normal and transformed cells, and methods for producing biologically active mature TGF-β in eucaryotic cells have been described (2, 37, 8, 11, 38).

R. Derynck et al. (38) have described a method comprising (a) constructing a vector which includes nucleic acid encoding TGF-β3, (b) transforming a heterologous host eucaryotic cell with the vector, (c) culturing the transformed cell, and (d) recovering TGF-β3 from the culture medium.

Summary of the Invention

The present invention provides methods for producing and purifying proteins comprising TGF-β3 precursor, pro region f the TGF-β3 precursor and mature TGF-β3. Moreover, this invention also provides methods for producing and purifying novel homodimeric proteins with enhanced production and heightened therapeutic utility which comprises mutations, signal peptide deletions, and protease cleavage site substitutions.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence encoding TGF-β3 and its deduced amino acid sequence. Putative glycosylation sites and polyadenylation signal are underlined. The start of the mature TGF-β3 is marked by an asterisk at alanine at nucleotide positions 1163-1165.

Figure 2 shows a Southern blot analysis of human tumor DNAs hybridized with a PvuII-PvuII TGF-β1 cDNA probe.

Figure 3 shows a Northern blot analysis of A673, A549, and A498 cell lines using an EcoRI-BglII 1.7kb cDNA fragment of the gene encoding TGF-β3 as a probe.

Figure 4 shows a Northern blot analysis of A673, A549, and A498 cell lines using a PvuII-TaqI probe from genomic sequences of TGF-β3.

Figure 5 shows a Northern blot analysis of A673, A549, and A498 cell line using a PstI-BalI TGF-β1 probe.

Figure 6 shows a Northern blot analysis of A673, A549, and A498 cell lines using TGF-β1 cDNA containing the complete coding sequence of TGF-β1 precursor as a probe.

Figure 7 shows a Northern blot analysis of mRNA from umbilical cord and A673 cell lines using an EcoRI-BglII cDNA fragment f the gene encoding TGF-β3 as a probe.

Figure 8 shows the production of trpE::TGF-β3 fusion protein of three lysates by SDS polyacrylamide gel electrophoresis. (A) corresponds to TGF-β3.

Figure 9 shows whole cell bacterial lysates containing

trpE::TGF-β1 fusi n prot ins (lanes 1 and 4), trpE::(A) fusi n proteins (lanes 2 and 5), and the TGF-β1 pr tein (purchased from R&D Systems) (lanes 3 and 6) were separated on a 12.5% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter (1 μm pore size) and incubated with 100 μg of affinity purified anti-peptide antibody either in the absence (lanes 1, 2 and 3) or presence of a 300 fold molar excess of the antigenic peptide (lanes 4, 5, and 6). The antibodies were detected using alkaline phosphatase conjugated to goat anti-rabbit antibody (Promega) according to the manufacturers instruction.

Figure 10 shows a schematic diagram of mRNA encoding TGF-β3 with the coding sequence boxed. The relative extension of the cDNA inserts obtained from placenta (1.7 kb), umbilical cord (1.9 kb) and A673 (1.7 kb) libraries is indicated. The dashed part of the box represents the C-terminal region showing high homology to TGF-βs. The 5' EcoRI-Bg II restriction fragment of the placenta cDNA is indicated by a bar.

Figure 11 A/B shows a comparison of the nucleotide sequence and predicted amino acid sequence of the gene encoding TGF-β3 with TGF-β1 and TGF-β2. Identical amino acids are boxed. The mature TGF-β3 amino acid sequences start at position 315. (A) corresponds to TGF-β3.

Figure 12 is a schematic representation of the construction of the pCMV-TGF-β3 expression plasmid from pORFX and pBlue-TGF-β3 plasmids.

Figure 13 shows the level of TGF-β3 mRNA expression, determined by Northern hybridization using a TGF-β3 specific probe, of parental CHO cells (lane 1), CHO cells transfected with TGF-β3 cDNA (CHO 6.35) (lane 2) and CHO 6.35 amplified with 20nM MTX (CHO 6.35/20nM (lane 3).

Figur 14
(A) shows the dose response of mink cell growth inhibition using purified TGF-β1. Cell growth was quantitated by the metabolism of MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetraazolium bromide; Thiazolyl blue) (148).
(B) shows the dose response of mink cell growth inhibition using acid activation serum free supernatants CHO 6.35/20nM transfectant and CHO 6.35 transfectant. Cell growth was quantitated by the metabolism of MTT.

Figure 15 shows the relative location of the various TGF-β3 peptides used as antigens.

Figure 16 shows the immunoprecipitation of native recombinant TGF-β3 protein by β3V antibody.

Figure 17
(A) shows the immunoblot of TGF-β3 from conditioned media f CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection from gels under reducing conditions.
(B) shows the immunoblot of TGF-β3 from conditioned media of CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection from gels under non-reducing conditions.

Figure 18 shows a Western blot of cell extract (18A) and conditioned media (18B) of the CHO 6.35/20nM transfectant using β3V antibody for detection.

Figure 19 A,B,C,D shows the staining to paraffin sections of human umbilical cord by β3V antibody and control antibody. A and C show fibroblast and epithelial staining and smooth muscle fiber staining, respectively, by β3V antibody. B and D show no staining by control rabbit polyclonal antibody.

Figure 20 is a silver stained gel f purified TGF-β3 and TGF-β1.

Figure 21 A, B, C shows specific antibody neutralization of TGF-β3 inhibition of mink cell growth by β3V.

Figure 22 shows the location of the various protease sites genetically engineered into the TGF-β3 precursor.

Detailed Description of the Invention

In accordance with the invention, mature TGF-β3 is defined as a recombinant homodimeric protein which comprises two polypeptides each of which consists essentially of 112 amino acids and has a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498.

Moreover, as used herein TGF-β3 precursor is a recombinant homodimeric protein which comprises two polypeptides, each polypeptide encoded by a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a serine encoded by nucleotides 1496-1498.

Further, as used herein the pro region of the TGF-β3 precursor is a recombinant protein which comprises the TGF-β3 precursor without the mature TGF-β. In particular, the pro region of the TGF-β3 precursor is a protein encoded by a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an methionine encoded by nucleotides 263-265 and ending with a arginine encoded by nucleotides 1160-1162.

Also, as used herein, reference to TGF-β means either mature TGF-β (e.g. TGF-β1, -β2, -β3), TGF-β precursor (e.g. TGF-β1 precursor, TGF-β2 precursor, TGF-β3 precursor), or the pro region of the TGF-β (e.g. TGF-β1, -β2, -β3) precursor.

The present invention provides a method of recovering purified, non-denatured mature TGF-β3 from a mixture of mammalian cell-derived polypeptides. The method comprises contacting the mixture with an antibody which specifically

binds to mature TGF-β3 but exhibits substantially no cr ss r activity with mature TGF-β1 and matur TGF-β2.

In one example, the mixture of mammalian cell-derived polypeptides is a mixture of non-human mammalian polypeptides from non-human cells in which TGF-β3 has been expressed.

Additionally, in another example of the subject invention, the antibody may be directed to an epitope defined by the amino acid sequence YLRSADTTHSTVLGLYNTLNPEASASY. By way of example, the previously described antibody may be immobilized on a solid support under conditions such that TGF-β3 is isolated and purified.

Additionally, this invention provides a method for producing substantially purified TGF-β3 precursor having an anchorage membrane sequence which comprises: (a) preparing DNA encoding a TGF-β3 precursor having the membrane anchorage sequence; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF-β3 precursor is expressed and subsequent translocation of the expressed TGF-β3 precursor having the membrane anchorage sequence; (d) culturing the host cell in medium; (e) separating the host cell from the medium; (f) disrupting the cell such that a lysate containing the TGF-β3 precursor having the membrane anchorage sequence is produced; and (g) purifying the TGF-β3 precursor having a membrane anchorage sequence from the lysate under conditions such that the substantially purified TGF-β3 precursor is produced.

The present invention also provides a method for producing substantially purified TGF-β3 precursor having an anchorage membrane sequ nc . The method c mprises: (a) preparing DNA

encoding the TGF-β3 precursor having a membrane anchorage sequence; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF-β3 precursor is expressed and subsequent occlusion of the expressed TGF-β3 precursor having a membrane anchorage sequence in occlusion bodies; (d) culturing the host cell in culture medium; (e) separating the occlusion bodies from the host cells and the culture medium; (f) disrupting the occlusion bodies to produce a solution containing the TGF-β3 precursor having a membrane anchorage sequence; and (g) purifying the resulting TGF-β3 precursor having a membrane anchorage sequence from the lysate under conditions such that the substantially purified TGF-β3 precursor is produced. The above-described method further comprises: (a) treating the purified TGF-β3 precursor s recovered with an activating agent to separate a mature TGF-β3 from the precursor; and (b) recovering the separated mature TGF-β3 of step (a).

In accordance with the present invention, the anchorage linkage sequence may be a phosphatidyl inositol linkage. Alternatively, the anchorage linkage sequence is a hydrophobic transmembrane peptide sequence.

Also, the present invention provides a method for producing a substantially purified mutant TGF-β3 precursor. The method comprises (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence

substantially identical t the amino acid sequence sh wn in Figur 1 beginning with a alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell of step (b) is transformed with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF-β3 precursor is expressed; (d) culturing the host cell in medium under conditions such that the mutant TGF-β3 precursor so expressed is secreted into the medium; (e) separating the host cell from the culture medium containing the mutant TGF-β3 precursor so secreted; and (f) purifying the mutant TGF-β3 precursor such that a substantially purified mutant TGF-β3 precursor is produced. The above-described method further comprises: (a) treating the purified mutant TGF-β3 precursor so recovered with an activating agent to separate a mature TGF-β3 from the precursor; and (b) recovering the separated mature TGF-β3 of step (a).

Moreover, this invention also provides a method for producing a substantially purified mutant TGF-β3 precursor. The method comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498; inserting the DNA f step (a) int an expressi n vector linked to a suitable promot r compatible with a host

cell; (c) transforming the h st cell with the vector in order to induce expressi n f the DNA f step (b) such that a mutant TGF-β3 is expressed; (d) separating the h st cell containing the expressed, mutant TGF-β3 precursor from the culture medium; (e) disrupting the host cell such that a lysate containing the mutant TGF-β3 precursor is produced and (f) purifying the mutant TGF-β3 precursor such that a substantially purified mutant TGF-β3 precursor is produced. Additionally, the above-described method further comprises (a) treating the purified mutant TGF-β3 precursor so recover d with an activating agent to separate a mature TGF-β3 from the precursor and (b) recovering the separated mature TGF-β3 f step (a).

Additionally, this invention provides a method for producing a substantially purified mutant TGF-β3 precursor. The method comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a alanine encoded by nucleotides 1163-1165 and ending with a asparagin encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) int an expression vector linked to a suitable promoter compatible with a h st cell; (c) transf rming the host cell with the

vector in order to induce expression of the DNA of step (b) such that a mutant TGF-β3 precursor is expressed; (d) culturing the host cell in medium under conditions such that the expressed mutant TGF-β3 precursor is secreted into the medium; (e) separating the cell from the culture medium containing the mutant TGF-β3 precursor so secreted; and (f) purifying the mutant TGF-β3 precursor such that a substantially purified mutant TGF-β3 precursor is produced. The method further comprises: (a) treating the purified mutant TGF-β3 precursor so recovered with cyanogen bromide to separate a mature TGF-β3 from the precursor; and (b) recovering the separated mature TGF-β3 of step (a).

Further, the invention also provides a method for producing a substantially purified mutant TGF-β3 precursor which comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a alanine encoded by nucleotides 1163-1165 and ending with a asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b)

such that a mutant TGF-β3 precursor is expressed; (d) separating the host cell containing the expressed, mutant TGF-β3 precursor from the culture medium; (e) disrupting the host cell such that a lysate containing a mutant TGF-β3 precursor is produced; and (f) purifying the mutant TGF-β3 precursor such that a substantially purified mutant TGF-β3 precursor is produced. The method further comprises: (a) treating the purified mutant TGF-β3 precursor so recovered with cyanogen bromide to separate a mature TGF-β3 from the precursor; and (b) recovering the separated mature TGF-β3 of step (a).

Further, in accordance with the practice of the above-described methods, X in step (a) may be selected from a group of tri-nucleotides consisting of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT, TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT, CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, ATA, ACT, ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG, GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA, GAG, GGT, GGC, GGA, and GGG.

In one embodiment of the subject invention, purification is effected by affinity chromatography. One example of affinity chromatography is antibody column chromatography. Another example of affinity chromatography is lectin column chromatography. Lectin column chromatography allows isolation of the glycosylated precursor form of TGF-β3.

Also, in one example of the invention, in step (a) of the above-described method, the protease cleavage sequence may be a collagenase recognition sequence. Alternatively, the protease cleavage sequence may be a Factor Xa recognition sequence. The protease cleavage sequence allows for the production of a membrane anchored TGF-β3 precursor.

This invention also provides a method for producing mutant

TGF-β3. The method comprises (a) preparing a DNA comprising a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with leucine encoded by nucleotides 332-334 and ending with serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector operably linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF-β3 is expressed; (d) culturing the host cell in medium; (e) separating the host cells containing the mutant TGF-β3 so expressed from the medium; (f) disrupting the cells to produce a lysate containing the mutant TGF-β3; and (g) purifying the mutant TGF-β3. The above-described method further comprises (a) treating the purified mutant TGF-β3 so recovered with an activating agent to separate a mature TGF-β3 from the mutant TGF-β3; and (b) recovering the separated mature TGF-β3 of step (a).

This invention additionally provides a process which comprises: (a) contacting a TGF-β3 precursor with a precipitating agent thereby concentrating the TGF-β3 precursor in a precipitate; (b) extracting the pellet of step (a) with an acidified organic solution under such conditions that mature TGF-β3 is separated from the pellet; and (c) recovering the mature TGF-β3 so separated in step (b).

In accordance with the subject invention, the acidified organic solution in step (b) may be an acidified acetonitrile. Additionally, the organic solution may comprise 50% acetonitrile and 1.0M acetic acid. Also in accordance with the subject invention, the precipitating agent in step (a) may be ammonium sulfate.

This invention also provides a method for producing and identifying a mutant, mature TGF-β3 exhibiting reduced binding

affinity to serum binding proteins. The method comprises (a) preparing a DNA encoding the TGF-β3; (b) performing mutagenesis (for example random mutagenesis) on the DNA of step (a) thereby obtaining a mutant DNA; (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell; (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF-β3 is expressed; (e) culturing the host cell in medium under conditions such that the expressed mutant TGF-β3 is secreted into the medium; (f) separating the host cell from the culture medium containing the mutant TGF-β3 so expressed; (g) purifying the mutant TGF-β3; (h) activating the mutant TGF-β3 so expressed under conditions such that a mutant, mature TGF-β3 is separated from the mutant TGF-β3; and (i) assaying the culture medium for the mutant mature TGF-β3 thereby identifying a mutant mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins.

Additionally, this invention further provides a method for producing and identifying a mutant, mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins. The method comprises: (a) preparing a DNA encoding the TGF-β3; (b) performing mutagenesis (for example random mutagenesis) on the DNA of step (a) thereby obtaining a mutant DNA; (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell; (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF-β3 is expressed; (e) culturing the host cell in medium under conditions such that the expressed mutant TGF-β3 is produced in the host cell; (f) separating the host cell containing the mutant TGF-β3 so expressed from the culture medium; (g)disrupting the cells to produce a lysate containing the mutant TGF-β3; (h) purifying the mutant TGF-β3;

(i) activating the mutant TGF-β3 so expressed under conditions such that a mature TGF-β3 is separated from the mutant TGF-β3; and (j) assaying the culture medium for the mutant mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins thereby identifying a mutant, mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins.

In one example of the above-described method, the serum binding protein is α2-macroglobulin. Alternatively, in another example, the serum binding protein is type III TGF-β receptor, e.g. betaglycan. Further alternatively, the serum binding protein may be a pro region of the TGF-β precursor.

This invention further provides a method for producing a substantially purified pro region of the TGF-β3 precursor. The method comprises: (a) preparing DNA encoding the pro region of the TGF-β3 precursor; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF-β3 precursor is expressed; (d) culturing the host cell in medium; (e) separating host cells from the medium; (f) disrupting the host cells to produce a lysate containing the pro region of the TGF-β3 precursor; and (g) purifying the pro region of the TGF-β3 precursor from the lysate such that the substantially purified pro region of the TGF-β3 precursor is produced.

Additionally, this invention provides a method for producing a substantially purified pro region of the TGF-β3 precursor which comprises: (a) preparing DNA encoding the pro region of the TGF-β3 precursor; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions

such that a pr regi n of the TGF-β3 pr curs r is expressed; (d) culturing the host c ll in medium under conditions such that the expressed pr region f the TGF-β3 precursor is secreted into the medium; (e) separating host cells from the medium containing the pro region of the TGF-β3 precursor so secreted; and

(f) purifying the pro region of the TGF-β3 precursor such that the substantially purified pro region of the TGF-β3 precursor is produced.

Further, in each of the above-described methods, the host cell may be either a eucaryotic cell or a procaryotic cell.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS

Abbreviations and Technical Terms:

AL (acute leukemia)
ANLL (adult non-lymphocytic leukemia)
APRT (adenosylphosphoribosyl transferase)
BFU-E (burst forming unit-erythroid)
BSA (bovine serum albumin)
CL (chronic leukemia)
CLL (chronic lymphocytic leukemia)
CML (chronic myelogenous leukemia)
CNBr (cyanogen bromide)
CFU (colony forming unit)
CFU-E (colony forming unit-erythroid)
CFU-GEMM (colony forming unit-granulocyte, erythroid, macrophage, monocyte)
CFU-GM (colony forming unit-granulocyte/macrophage)
CFU-meg (colony forming unit-megakaryocyte)
CHO (chinese hamster ovary)
CMV (cytomegalovirus)
CSF (colony stimulating factor)
DHFR (dihydrofolate reductase)
DMEM (Dulbecco's modified Eagle's medium)
DMF (dimethyl formamide)
DMSO (dimethyl sulfoxide)
DNA (deoxyribonucleic acid)
EPO (erythropoietin)
FCS (fetal calf serum)
G-CSF (granulocyte-colony stimulating factor)
GM-CSF (granulocyte/macrophage-colony stimulating factor)
kb (kilobase pairs)
kDa (kil Daltons)
HPLC (high pressure liquid chromatography)

IL-3 (interleukin-3)
IL-4 (interleukin-4)
MEM (modified Eagle's medium)
mRNA (messenger ribonucleic acid)
RNA (ribonucleic acid)
TGF-ß (transforming growth factor-beta)
TIF (tumor inhibitory factor)
WBC (white blood cell)

EXAMPLE 1: CLONING THE GENE ENCODING TGF-β3

To identify sequences with homology to TGF-β1 a Pvu II-Pvu II probe, containing most of the mature form of the TGF-β1 cDNA sequence, was ^{32}P labelled and used to screen a Southern bl t (34) of total human DNAs digested with Eco RI, Hind III or Sst I using standard methods. In each digest, two bands were present at a low stringency wash (2.5 x SSC, 65°C) (Figure 2). When the wash stringency was increased (0.01 x SSC, 65°C) only one hybridizing band remained in each digest (Figure 2). Th strongly hybridizing band is TGF-β1, and the weakly hybridizing band is a related gene which also encodes TGF β3. The nucleotide sequence encoding TGF-ß3 and its amino acid sequence are shown in (Figure 1).

To isolate the gene encoding TGF-ß3 with homology to TGF-β1, the TGF-β1 clone was used to screen a human phage library constructed from the DNA of a chronic myelocytic leukemia cell line (K562). Two genomic loci, which correspond to TGF-β1 and the related gene encoding TGF-ß3 (Figure 1), were cloned. Construction of the K562 library, screening and isolation of recombinant clones was carried out essentially according to the procedures of Grosveld, et al. (15).

The phage DNA cl ne c ntaining the sequence encoding TGF β3, was cut with Sau 3A and the restricti n fragments cloned into

M13. The recombinant plaques were screened with the SmaI-PvuII probe of TGF-β1. Six hybridizing genomic clones were sequenced by the method of Sanger, et al. (33) and a region of approximately 130 bp was found to be homologous to TGF-β1 cDNA. When the amino acid sequence of TGFβ1 and TGF β3 cloned in these experiments were compared they were found to be 82% homologous.

To obtain a repeat free probe of TGF-β3 various restriction fragments from BamHI-BamHI subclone of this gene were hybridized to TGF-β1 cDNA, as well as to total human DNA. A BamHI-TaqI fragment of the gene, i.e. TGF β3, was found to cross hybridize with the TGF-β1 cDNA but did not hybridize to repetitive sequence elements in human DNA.

The BamHI-TaqI unique probe of the sequence encoding TGF-β3 was used to screen the lambda-gt11 human placenta cDNA library (Clonetech). Two strongly hybridizing clones, as well as four weakly hybridizing clones, were isolated. By DNA sequence analysis the weakly hybridizing clones were shown to correspond to TGF-β1. One strongly hybridizing clone was isolated and a 1.7kb EcoRI insert was subcloned into pUC 8.

Restriction fragments for this clone were subcloned into M13 and sequenced by the method of Sanger, et al. The deduced amino acid sequence of this gene exhibits extensive homology with a family of genes (24) including TGF-β1, TGF-β2, glioblastoma T-cell suppressor factor (G-TsF), inhibin/activin, Mullerian Inhibiting Substance (MIS) and decapentaplegic transcript complex of Drosophila with the six C-terminal cysteine residues being conserved throughout.

A 17 kb genomic DNA fragment containing the sequence of the gene, i.e. TGF-β3, has been cloned. Hybridizing 5' and 3' portions of the 1.7 kb cDNA clone which encodes TGF-β3 with

the genomic locus of TGF-β3 revealed that the 1.7 kb cDNA sequence is completely contained in the genomic clon . Taking into account that the full length message of TGF-β3 is 3.5 kb, additional 5' and 3' flanking sequences need to be isolated to obtain the complete gene. This is done by screening genomic phage and cosmid libraries with probes unique to the gene encoding TGF-β3.

In TGF-β1 the sequence R-R represents the proteolytic cleavag site which generates the mature protein. In TGF-β3 the sequence R-K-K-R represents the corresponding cleavage site.

In the region N-terminal to the cleavage site, TGF-β1 and TGF-β3 exhibit only 35% homology. However, both proteins contain the sequence R-G-D in the N-terminal region which is recognized by the fibronectin receptor.

In order to determine which cell types express TGF-β3, Northern hybridization was carried out using a 5' terminal Eco RI-Bgl II probe (Figure 3). The result of Northern hybridization revealed a mRNA of approximately 3.5 kb in A673 (a rhabdomyosarcoma), A498 (a kidney carcinoma) and a faintly hybridizing signal in A549 (a lung adenocarcinoma).

A genomic probe from the 3' region of TGF β3 (corresponding t sequences downstream of the presumed site of proteolytic cleavage) was then used to screen the same Northern blot. Three strong hybridization signals were observed in both A673 and A498, corresponding to TGF-β1 (2.5 kb), TGF-β3 (3.5 kb) and another related gene (4.2 kb) (Figure 4). These results are consistent with the notion that this probe cross reacts with sequences homologous to TGF-β3.

Northern blot analysis of A673, A549 and A498 cell lines using a PstI-BalI TGF-β1 pr be was then perf rmed. The PstI-BalI

TGF-β1 probe strongly hybridized to a 2.5 kb mRNA band in all three cell lines. Several weakly hybridizing bands are also observed at 4.2 kb and 3.5 kb (Figure 5). This probe is highly specific for TGF-β1 since it contains sequences corresponding to those residues N-terminal to the proteolytic cleavage site, a region where TGF-β1 exhibits little homology to other members of this gene family.

A Northern blot of A673, A549 and A498 cell lines were then screened using TGF-β1 cDNA containing the complete coding sequence of the TGF-β1 precursor. This probe cross hybridized with homologous sequences to TGF-β1. Specifically, there was strong hybridization to a 2.5 kb mRNA band corresponding to TGF-β1 (Figure 6).

Northern blot analysis of mRNA from human umbilical cord and the A673 cell line was also screened using an EcoRI - BglII cDNA fragment of TGF-β3 as a probe (Figure 7). Figure 7 also illustrates the result of a Northern blot using an actin probe as a control to normalize mRNA levels in each lane. When normalized to actin mRNA levels, the umbilical cord expresses the highest level of mRNA of the gene encoding TGF-β3 in comparison to other mRNA sources so far examined.

Southern blot analysis was performed on a variety of different tumor DNAs digested with EcoRI and hybridized with a SmaI-AvaI cDNA fragment of TGF-β3 as a probe. Hybridization was effected at both low (2.5 X SSC, 65°C) and high (0.3 X SSC, 65°C) stringency washes. Southern blot analysis indicated the possible presence of other loci related to TGF-β3. The probe hybridized with two bands (3 kb and 12 kb) which were observed only if washed with low stringency.

Producti n of Antibodies with Specificity for TGF-ß3

Chimeric bacterial proteins, i.e. fusion proteins, have been constructed which contain the C-terminal 150 amino acids f TGF-ß3 fused to a small region of the trpE gene. The fusion protein was recognized by an antibody produced against a peptide derived from amino acid numbers 9 to 28 of the matur form of TGF-ß3. The antibody recognized the trpE::TGF-ß3 fusion protein and the peptide specifically competes with TGF-ß3 for the binding of the antibody.

DNA sequences that code for TGF-ß3 were cloned into a pKS vector. This vector is a pATH II derivative that contains the inducible trp promoter and a multiple cloning site. The resulting constructs produce a chimeric protein consisting f the first 22 amino acids of the trpE gene, the C-terminal 150 amino acids of TGF-ß3.

Transformants containing these clones were screened primarily by restriction endonuclease analysis and ultimately for production of the chimeric protein by SDS polyacrylamide gel electrophoresis. The protein products of 3 clones, p116, p134, and p135, are shown in Figure 8. These cells were grown in defined media until they reached early log phase and then incubated for 3 hours either in the presence or absence of th trpE inducer indoleacrylic acid (IAA). The cells were then collected, lysed and their proteins electrophoresed on a 12.5% SDS polyacrylamide gel. Figure 8 is a photograph of one such gel that had been stained with Coomassie blue. As can be seen, lysates p116 and p135 produce a protein of about 19,000 Dalton molecular weight whose relative abundance increases in the presence of IAA. In contrast, p134 does not produce this protein species. Both p116 and p135 contain plasmids that, by restricti n analysis, have the sequences of TGF-ß3 cloned in

the orientation that should produce a 19,500 Dalton molecular weight fusion protein. The p134 plasmid was found to have the sequences of TGF-β3 in the opposite orientation.

The trpE::TGF-β3 fusion protein was used to test the specificity of an antibody that used a peptide homologous to part of TGF-β3 as an antigen. A polypeptide was synthesized corresponding to residues 9 through 28 of mature TGF-β3 except that residue 9 in the sequence, arginine, was replaced by serine. The peptide was purified by reverse phase HPLC and coupled to keyhole limpet hemocyanin for use as an immunogen in rabbits.

Thirty-three days following the first injection (500 μg), the antisera were screened by standard ELISA using 100ng of peptide per well. One rabbit demonstrated a signal of 1.0 OD units at a 1:25 dilution of the antisera. Ten days after this rabbit was first bled, a boost of 250mg of coupled antigen was given. The following bleed 20 days after the first bleed showed a 20-fold increase in antibody response to the peptide antigen. Forty days after the initial bleed (3rd bleed) a signal of 1.0 OD unit was achieved at a 1:8000 dilution of the antibody, a 16-fold increase in antibody titer over the second bleed. This antibody showed little cross-reactivity with a homologous peptide derived from TGF-β1 sequences. The TGF-β1 derived peptide consisted of amino acid numbers 4 to 19 of the mature TGF-β1 protein. Of the 11 common amino acids, i.e. residues 9-19, 7 are conserved between TGF-β3 and TGF-β1.

To determine if the peptide recognizing antibody could recognize TGF-β3, the antibody was used in Western blot analysis against a trpE:mature TGF-β3 and trpE:mature TGF-β1 fusion proteins. As seen in Figure 9, the anti-peptide antibody reacted strongly with the fusion protein of TGF-β3 while it reacted only weakly with a trpE::TGF-β1 fusion

protein. Both fusion proteins were recognized by a commercially available anti-TGF-β1 antibody (R and D systems) (Figure 9).

As can be seen in Figure 9, the anti-peptide antibody recognizing TGF-β3 also has a high level of background reactivity to bacterial proteins. To reduce this cross reactivity, we purified the antibody on a CNBr-Sepharos column containing the original peptide used as an antigen. The antibody retained its high titer to the peptide of TGF-β3 and low cross reactivity to the homologous peptide derived from TGF-β1. The purified antibody reacts very strongly with the fusion protein of TGF-β3.

Eucaryotic Expression of TGF-β1 Fused With TGF-β3

Human recombinant TGF-β1 has been expressed in monkey COS cells. Sequences encoding the complete precursor of the TGF-β1 cDNA were cloned down stream from a SV40 promoter using the pSVL eukaryotic expression vector (obtained from Pharmacia). This construct was transfected into COS cells using a standard calcium phosphate precipitation method (13). After transfection, approximately 4 x 10^6 cells were grown in serum free media for two days. The conditioned media was then collected, acidified and tested for biological activity. Conditioned media from TGF-β1 transfected cells was found to inhibit the growth of a monolayer mink lung test cell line (CCL 64) by 59% as compared to conditioned media from COS cells transfected with the pSVL vector alone which inhibited growth of CCL 64 cells by only 32%.

A chimeric pro region of the TGF-β1 precursor::mature TGF-β3 fusion construct was made by substituting 5' sequences of the TGF-β1 precursor with sequences encoding TGF-β3. Given the homology between the two proteins and the conserved position

of th ir cysteine residues, wh n such a construct is transf cted into COS cells th novel fusion pr tein is processed into the biologically active mature TGF-β3. Additional constructs, which consist of the trpE::TGF-β3 fusion cloned under the regulator sequences of either the SV40 promoter of the long terminal repeat of the mouse mammary tumor virus (MMTV) have been made and tested for biological activity in transient transfection experiments.

EXAMPLE 2: Further sequence determination of a gene encoding TGF-β3

Screening a lambda gt11 human placenta cDNA library (Clontech 1.2 x 10^6 independent clones) with a repeat free probe of the gene encoding TGF-β3, resulted in the isolation of a 1.7 kb cDNA clone. On Northern analysis, the mRNA for TGF-β3 was found to be approximately 3.5 kb, indicating that a full length cDNA had not been obtained.

To obtain additional 5' sequence information, a lambda gt11 human umbilical cord cDNA library (Clontech, 1.5 x 10^6 independent clones) was screened with a 5' EcoRI-BglII restriction fragment derived from the placenta cDNA clone. This resulted in the isolation of a 1.9 kb cDNA. Sequence analysis revealed this clone contained an additional 180 nucleotides of 5' sequence information. The isolation of this cDNA from an umbilical cord library again confirms that this gene is actively transcribed in this tissue.

To obtain further cDNA sequence information for the gene encoding TGF-β3, mRNA was isolated from A673 cells and a cDNA library prepared. Starting with 5 μg poly $(A)^+$ RNA, a random primed cDNA library of approximately 2 x 10^6 clones was constructed in lambda gt10, using the Amersham cDNA synthesis system plus according to the manufacturer's procedures.

Appr ximately 0.7 x 10^6 unamplified cDNA clones were screened with a 25-m r ligonucleotide pr be (5' ATATAGCGCTGTTTGGCAATGTGCT 3') corresponding to a sequence near the 5' end of the 1.9 kb cDNA clone and a single positive clone containing a 1.7 kb insert was identified.

Analysis of the three overlapping cDNAs (Figure 10) revealed a sequence of 2529 bases, with the largest open reading frame being 1236 bases. No sequence difference were found in the overlapping cDNAs indicating they were derived fr m transcripts of the same gene. Overlapping sequences comprise a complete 3' untranslated region of 1031 bp with a polyadenylation signal 25 bp upstream from the poly(A) tract. The 5' untranslated region comprises 262 bp but lacks approximately 1 kb, as judged from the size of the mRNA estimated by Northern analysis. The predicted amino acid sequence of the gene encoding TGF-β3 shows extensive homology to TGF-β1 and β2 (Figure 11) (8, 9, 22).

TGF-β1 and TGF-β2 are produced in precursor forms of 390 and 414 amino acid residues respectively (8, 9). The cDNA sequence obtained for the gene, i.e. TGF-β3, encoding TGF-β3 (Figure 1) contains an open reading frame coding for 412 amin acids, with the first ATG preceded by a stop codon, 162 nucleotides upstream. As found with TGF-β1 (10), the initiating codon for the protein having tumor inhibitory activity does not form part of a Kozak consensus (20).

Interestingly, six nucleotides downstream there is a second ATG, with an A at position -3, which aligns with the initiating codon in TGF-β2 (9). Homodimers of the C-terminal 112 residues of TGF-β1 and β2 represent the biologically active forms of these proteins. Preceding the site of cleavage to their mature forms, TGF-β1 and -β2 have stretches of 4 and 5 basic residues, respectively. In TGF-β3 there are

5 basic residues preceding th cleavage site marked by th ast risk (Figur 10). The mature f rms of TGF-β1 and -β2 share 80/112 identical residues. The corresponding 112 C-terminal amino acids in TGF-β3 exhibit 86/112 and 89/112 identical residues compared to TGF-β1 and -β2, respectively (Figure 11). Many of the remaining differences represent conservative substitutions. All three proteins show a strict conservation of the cysteine residues in this region. The N-terminal domain of TGF-β3 precursor exhibits approximately 35% homology to TGF-β1 and 45% homology to TGF-β2. By comparison, the corresponding sequences of the TGF-β1 and -β2 precursors have 33% sequence homology (Figure 11) (8, 9). Four potential glycosylation sites are contained in the N-terminal part of TGF-β3 precursor, one of which is contained in all three proteins. All three proteins also possess hydrophobic N-termini which may represent presecretory signal peptide sequences (31). Interestingly, both TGF-β1 and TGF-β3 (but not TGF-β2) contain the fibronectin binding sequence R-G-D (32). By analogy to TGF-β1 and -β2, -β3 is synthesized a TGF-β3 precursor which undergoes proteolytic cleavage to produce the mature polypeptide. Based on the functional and structural homology to TGF-β1 and -β2, -β3 likely has therapeutic activity in cancer therapy, wound healing and immunosuppression.

EXAMPLE 3: EXPRESSION OF TGF-β3

TGF-β3 Expression Construct

A 1500 bp AluI-HgaI restriction fragment of TGF-β3 cDNA (sites are indicated in Figure 1) which encodes the complete TGF-β3 protein was cloned into the Bluescript plasmid (Strategene, La Jolla, CA) to yield the plasmid pBlue-TGF-β3. The f1 intergenic region of this vector allows the production of single stranded DNA via inf ction of its host bacteria with f1

helper phage. The initiation codon of TGF-β3 does not form part of a Kozak consensus sequence (CCACC[ATG]G) (20), which has been shown to influence the efficiency of translation. In order to promote high yields of the recombinant TGF-β3 protein, the flanking sequence of the initiation codon was mutagenized to a more efficient translation sequence by changing CACAC[ATG]A into CCACC[ATG]A using the method of Nakamaye and Eckstein (26). Mutagenesis was confirmed by sequence analysis. Expression yields are further optimized by deletion of TGF-β3 5' and 3' untranslated [non-coding] sequences. Subsequently, the mutagenized pBlue-TGF-β3 was cut with KpnI and SpeI, two polylinker restriction sites flanking the cDNA insert. This fragment was cloned into the eukaroytic expression vector pORFEX (3) cut with KpnI and XbaI. In this construct (pCMV:TGF-β3) the TGF-β3 cDNA sequence is transcriptionally regulated by the cytomegalovirus immediate early promoter (Figure 12).

DNA Transfection and Gene Amplification

Stable transformants expressing TGF-β3 were obtained by cotransfecting the pCMV-TGF-β3 construct (Figure 12) with the dihydrofolate reductase (DHFR) gene (the pDCHIP plasmid containing hamster DHFR minigene driven by its own promoter) into Chinese Hamster Ovary (CHO) cells, which lack the DHFR gene (35).

A standard $CaPO_4$.DNA precipitation method (13) was used for DNA transfection. pCMV:TGF-β3 (5.7 kb) and pDCHIP (2.5 kb) were coprecipitated with $CaPO_4$ in a ratio of 10 μg to 50ng respectively and the precipitate added to 0.5 X 10^6 CHO(DHFR-) cells. Selection of transformants with a DHFR+ phenotype was performed in alpha MEM (Gibco, Grand Island, NY) supplemented with 10% dialyzed fetal calf serum. The colonies that appeared after culturing for 10-14 days in selection medium

were isolated by standard methods and xpanded.

For gene amplification, the primary transfectants were subjected to stepwise selection with increasing concentrations of methotrexate (MTX; Sigma Chemical Co., St. Louis, MO). The first round of selection was carried out at 20nM MTX. TGF-β3 expression levels were measured by RNA cytodot hybridization normalizing the expression of TGF-β3 mRNA to that of actin. Two of the three clones with initial high expression (clones CHO 6.35 and CHO 9.1) showed increased TGF-β3 mRNA expression at 20nM MTX concentration (Figure 13). Total RNA (75 μg) from CHO cells (lanes 1), CHO 6.35 (lane 2), and CHO 6.35/20nM (lane 3), were fractionated on a 1.2% agarose-formaldehyd gel, blotted onto nitrocellulose and probed with a TGF-β3 specific probe (EcoRI-SmaI cDNA restriction fragment of a partial TGF-β3 cDNA clone isolated from umbilical cord; see Figure 10). CHO 6.35/20nM (primary transfectant CHO clone 6.35 at 20nM MTX), which had the highest level of expression, was chosen for initial protein purification from conditioned media and for further gene amplification.

The best clone from further MTX selection (10 μM MTX) was expanded and a bank of frozen stocks established. This clone, 6.36H, was used in all subsequent production of TGF-β3 and was maintained in T225 flasks (225 cm^2) in alpha MEM supplemented with 10% dialyzed fetal bovine serum. TGF-β3 production involved seeding Nunc cell factories (6000 cm^2 of surface area per factory), with cells from three confluent T225 flasks of 6.35H in alpha MEM supplemented with 10% dialyzed FBS. The cells were allowed to grow to 80% confluence in the cell factories. Media was then replaced with HB CHO, a serum-free media from HANA (Hana Biologics). After 72 hours, media was removed and replaced with fresh HB CHO for a total of 5 collections of conditioned HB CHO media. The first collection of c nditioned HB CHO m dia c ntained l w levels of TGF-β3

with the maximum amounts produced in the 4th thr ugh 6th c ll cti ns. Nunc cell fact ries provide sufficient surface area for the large scale growth of monolayer cell lines such as CHO, yielding a total of 7.5 liters of conditioned media per factor (3 collections, 2.5 liters per collection) with acceptable ease of use in a sterile environment. Using mor advanced expression vector systems, it should be possible by one skilled in the art to significantly increase production yields.

Alternatively, cell lines could be adapted to suspensi n growth and produced in either a stirred tank fermentati n system or in an air lift fermentator. The use of porous glass cylinder supports as a means of adapting monolayer cells (i.e. CHO cells) to stirred or air-life suspension culture has als been evaluated and shown to give acceptable yields of TGF-β3.

Expression of a mutant TGF-β3 Precursor

The mutant TGF-β3 precursor is expressed as a single homodimeric polypeptide in a host cell by mutation of the R-K-K-R cleavage site between the TGF-β3 pro region and mature TGF-β3 to a protease cleavage site, e.g. factor Xa cleavage sequence (Ile-Glu-Gly-Arg) or a collagenase cleavage sequenc (Pro-X-Gly-Pro) (Figure 22) using standard site directed mutagenesis procedures, followed by insertion of the mutant TGF-β3 nucleic acid into an expression vector and transfection of the mutant TGF-β3/vector DNA into a host cell (e.g. E. coli, any mammalian cells, e.g. CHO or HeLa cells, non-mammalian vertebrate cells, e.g. chick cells, and invertebrate cells, e.g. insect cells) together with DNA encoding a selectible marker (e.g. neo, dhfr).

Additionally, a mutant TGF-β3 precursor can be produced by preparing DNA comprising a first DNA sequence encoding an

amino acid sequenc substantially id ntical to th amino acid sequ nce shown in Figure 1 beginning with a methi nine ncod d by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising any tri-nucleotide sequence from the list consisting of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT, TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT, CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, ATA, ACT, ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG, GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA, GAG, GGT, GGC, GGA, and GGG. The DNA also comprises a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498. Such DNA may be inserted in a suitable expression vector and transfected into a host cell along with a selectible marker (either linked or unlinked to the expression plasmid) by one ordinarily skilled in the art.

Transfected cells may be cultured in an appropriate medium selecting for cells which express a selectible marker and such cells are further characterized for expression of mutant TGF-β3. Cells derived in this way may be used to produce mutant TGF-β3 for subsequent purification. Mature TGF-β3 may be released from the precursor by proteolytic cleavage when a protease cleavage site separates the mature and the pro region of the TGF-β3 precursor. Similarly, cyanogen bromide treatment may be used t releas mutant, mature TGF-β3 when

m thi nine separates the mutant, matur TGF-β3 lacking methionine at nucle tid s 1472-1474 and the pro regi n f the TGF-β3 precursor.

Biological Assay for Conditioned Media

Conditioned media was treated with acetic acid to a final concentration of 0.1 M and serial dilutions tested for biological activity. CCL 64, a cell line derived from Mink lung (American Type Culture Collection, Rockville, MD), was found to be extremely sensitive to the naturally occurring TGF-β3 isolated from umbilical cord. This cell line was initially chosen, therefore, to test conditioned media for biological activity of the recombinant TGF-β3 protein according to the method of Iwata, et al. (19). Growth inhibition of CCL 64 mink lung cells produced by TGF-β1 (purified) or TGF-β3 (from conditioned media) is shown in Figure 14 A/B.

Figure 14A shows a dose response of growth inhibition using purified TGF-β1 (Calbiochem); a 50% inhibition was obtained with 0.1ng TGF-β1. An increase in mink cell growth inhibitory activity was found comparing conditioned media form the transfectant selected at 20nM MTX versus media from th parental transfectant. Figure 14B shows the biological activity of acid activated serum free supernatants of CHO 6.35/20 nM transfectant (closed circles) and CHO 6.35 transfectant (open circles); 50% inhibition was obtained equivalent to 30 and 5 ng/ml TGF-β1 activity, respectively. Conditioned medium from parental CHO (DHFR-) possessed much lower growth inhibition than either transfectant. These results clearly show that the TGF-β3 cDNA is transcribed and that TGF-β3 mRNA is translated and produces biologically active protein.

In the presence of EGF, acidified conditioned media from CHO 6.35, containing TGF-β3 was able to promote soft agar growth of NRK cells. Growth of NRK cells in soft agar has been shown to be inducible by stimulating the production of extracellular matrix proteins, an important parameter in wound healing.

Immunodetection

Peptides corresponding to various partial amino acid sequences of the TGF-β3 protein were synthesized on an Applied Biosystems peptide synthesizer (Model 430A) using tBoc chemistry (Figure 15). Peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and used for immunization of rabbits. Enzyme-linked immunosorbent assays were used initially to characterize the antibody titers (Table 1). For this, and the following immunological experiments, standard techniques were employed (17). High titer antibody from immunized rabbits injected with β3V or β3III peptides were purified using an affinity matrix composed of the respective peptide β3 antigen conjugated to Affi-prep 10 (Bi Rad, Richmond, CA).

TABLE 1

Peptide	Sequence	Elisa Titer
I	EEMHGEREEGCTQENTESEY	1:6,000
IIL	GDILENIHEVMEIKRKGVDNEDD	1:10,000
IIs	GDILENIHEVMEIK	1:19,000
III	DTNYCFRNLEENC	1:26,000
IV	CVRPLYIDFRQDLGWKWVHEPKGYYANFC	1:19,000
V	YLRSADTTHSTVLGLYNTLNPEASASY	1:26,000
VI	CVPQDLEPLTILYYVGRTPKVEQLSNMVVKSC	1:4,000

The affinity purified β3III antibody exhibits greater than 300 f ld specificity for the β3III peptide c mpared t the c gnat peptide sequences from either the TGF-β1 or -β2. Furthermore, no significant cross reactivity of this antibody has been observed against either the TGF-β1 or -β2 proteins. However, this antibody shows only a very limited ability to immunoprecipitate the native recombinant TGF-β3 protein from conditioned media. The affinity purified β3V antibody exhibits at least a 400-fold selectively for the β3V peptid compared to the corresponding peptide sequence form TGF-β1. This antibody can also efficiently immunoprecipitate the native TGF-β3 protein (**Figure** 16).

Figure 17 A/B show an immunoblot of TGF-β3 in conditioned media produced by the CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection. For peptide blocking experiments, the antibody was preincubated with 80-fold molar excess of peptide prior to incubation with the blot. For detection, alkaline phosphatase (Zymed, San Francisco, CA) conjugated to goat anti-rabbit IgG was used as a second antibody. **Figure** 17A shows a Western blot of a gel where th sample was subjected to reduction prior to electrophoresis while **Figure** 17B shows the Western blot of the sample under non-reducing conditions. In each figure, lanes 1-3 and 4-6 corresponds to conditioned media immunoblotted with β3V and β3III antibody, respectively, lanes 2 and 5 immunoblots carried out in the presence of excess cognate peptide, while lanes 3 and 6 represent immunoblots in the presence of an excess unrelated peptide sequence.

Western blotting of conditioned media from CHO 6.35/20nM cells under reducing conditions, using affinity purified β3III and β3V antibody, detected a 50 kDa and a 12 kDa band. These bands correspond to the TGF-β3 precursor and mature TGF-β3, by analogy to the pr cessing f TGF-β1 and TGF-β2 described by

Gentry et al. (11) and Madisen et al. (22) (Figure 17A/B).

Under non-reducing conditions, 100 kDa and 24 kDa bands were observed, which we believe to correspond to homodimeric forms of the TGF-β3 precursor and mature TGF-β3. The apparent precursor appears as a broad band, characteristic of some glycosylated proteins. Following cleavage of the signal peptide sequence of the precursor form of TGF-β3, one would expect a protein with MW of 43 kDa (under reduced conditions).

Based on the primary sequence of TGF-β3, there are four N-linked glycosylation sites, further indicating that the detected precursor protein is glycosylated. Figure 18 A/B show Western blot of cell extract (Figure 18A) and conditioned media (Figure 18B) of the CHO 6.35/20nM transfectant using β3V antibody for detection. For preparation of cell extracts, cells were first washed with phosphate buffered saline then lysed directly with SDS/β-mercapthoethanol prior to gel electrophoresis. For peptide blocking (lanes 2 and 4), the antibody was incubated with a 100-fold molar excess of specific peptide prior to incubation with the blot (^{125}I protein-A was used for detection). In cell extracts of CHO 6.35/20nM under reducing conditions, only the 50 kDa band corresponding to a potential precursor form is detected (Figure 18 A/B). The specificity of the antibody was demonstrated by preabsorbing the antibodies with peptide immunogen prior to Western blotting (Figures 17 A/B and 18 A/B). As expected, based on mRNA and biological activity data, the antibody did not detect any TGF-β3 protein in conditioned media of the parental CHO (DHFR-) cells.

Both antibody were also tested for immunoprecipitation of native recombinant TGF-β3 protein (Figure 16). CHO 6.35/20nM w re grown to conflu ncy and labeled with [^{35}S] methionin for 24 hours in methionine-free DMEM in the presence of 5%

dialyzed plus 5% n n-dialyzed fetal calf serum. The medium was c llected and immun pr cipitated with 10 μg/ml affinity purified antibody and 20 μg/ml (1:2 dilution) protein A agarose, for 2 hours at 4°C. Separation of the immunoprecipitated proteins on a 12.5% SDS polyacrylamide gel revealed two proteins migrating identically to the mature TGF-β3 (12 kDa) and precursor TGF-β3 (50 kDa) (Figure 16). However, one extra immunoprecipitated protein was found at 43 kDa.

The 43 kDa protein may correspond to either the non-glycosylated precursor or a proteolytic breakdown product. The β3V antibody, in comparison to the β3III antibody, proved to be much more efficient in immunoprecipitating the TGF-β3 protein. The specificity of the immunoprecipitation was determined by preincubating the antibody with a 80-fold molar excess of either the cognate peptide or an unrelated peptide sequence. The specific peptide showed complete competition of all three bands whereas the unrelated peptide did not. As expected, based on the amino acid composition and distribution of methionine in the TGF-β3 protein, the 50 kDa contains significantly more ^{35}S label.

The β3V affinity purified antibody was also used in paraffin sections of human umbilical cord (Figures 19 A/B/C/D). Fibroblasts and epithelial cells stained (Figure 19A) as did the smooth muscle fibers of the cord vasculature (Figure 10C) whereas neither the connective tissue nor the extracellular matrix stained with this antibody. A control rabbit polyclonal antibody (Ig against P210phl/abl:OSI catalog #PC02) showed no staining (Figures 19 B and D). The strong staining in umbilical cord tissue agrees with earlier data showing extracts from umbilical cord possessed high levels of mRNA.

Preparati n of TGF-β3 Monocl nal Antibody

A TrpE-TGF-β3 fusion was produced in E. coli which had the foll wing charact ristics, i.e. amino acids 1 t 19 are c ded by the TrpE and poly linker segment and amino acids 20 to 170 correspond to amino acids 273 to 412 of the TGF-β3 precursor (containing the full mature TGF-β3 sequence). The fusion protein remained in the insoluble fraction after sonication in PBS. Subsequently, the protein was purified by separation on a SDS-polyacrylamide gel and isolated by electroelution. This material was used for immunization of mice by the following protocol:

a. Balb/C female mice were immunized intraperitoneally with 100μg of TrpE-TGF-β3 in RIBI adjuvant on days 0, 7 and 14;
b. On day 24 test bleeds indicated high titers against TrpE-TGF-β3 and purified TGF-β3 protein;
c. The mice were then boosted with 100μg of the same antigen on days 28, 29 and 30;
d. Spleen fusions were performed the following day; and
e. Subsequent methods of hybridoma selection, culture and subcloning were performed following standard procedures (17).

Five stable hybridomas were produced and their characteristics are shown in Table 2. All of the clones produced antibodies of the IgG k class. The monoclonal antibodies immunoblotted with purified TGF-β3. All five monoclonal antibodies showed no reactivity with TGF-β1 by ELISA, but crossreacted with TGF-β2.

Analysis of the epitopes recognized by the monoclonal antibodies using TGF-β3 synthetic peptides showed that all antibodies reacted with amino acids residu s 380 to 412.

EXAMPLE 4: A METHOD FOR PURIFICATION F TGF-β3 FROM CONDITIONED MEDIA

Conditioned medium was prepared from CHO 6.35/20nM cells grown to confluence in the presence of 20nM methotrexate. The cells were washed with phosphate buffered saline and incubated with serum free medium for 2 hours to eliminate carryover of serum proteins. Conditioned media was derived from cells incubated with fresh serum-free medium for 48 hours. TGF-β3 was purified from conditioned media using the following protocol.

i. Conditioned media was filtered through a 1 μm glass fiber filter (Micron Separations NC CG 20000-A01) and stored in plastic containers at 4°C after addition of 0.1mM PMSF, 4mM EDTA, 1mM EGTA, 0.02% sodium azide and 10mM Tris HCl pH 7.5.

ii. Media is concentrated approximately 100 fold using a high capacity, low protein binding Millipore "Pellicon" membrane cartridge (Millipore PLGC Regenerated Cellulose MW cutoff 10,000).

iii. The protein concentration was adjusted to 10 mg/ml and ammonium sulfate (90%, pH 7) is added to 45%, pH adjusted to 7.6, the material incubated for 4 hours at 0°C (or overnight). The precipitate pelleted by centrifugation (10,000 x g for 30 min) and the pellet left to drain for 10 minutes.

iv. The pellet was extracted with acetonitrile, 50% (v/v)/acetic acid (1M), at 0°C. 25 ml of extraction buffer was used per gram of starting protein in (iii) above. The suspension was treated t centrifugation at 10,000 x g f r 30 minutes.

v. The extraction supernatant was further concentrated using a Minitan concentrator (Millipore) using identical membranes as described in (ii). 1M acetic acid was added during concentration to prevent protein precipitation and change the buffer to 1M acetic acid.

vi. The concentrated material is chromatographed by P-60 gel filtration using a 1M acetic acid mobile phase, and peak fractions concentrated using the Minitan concentrator.

vii. The concentrate was made 1% in Triton X-100, the pH adjusted to 7.5 with solid Tris Base (Sigma) and clarified by centrifugation at 10,000 x g for 30 minutes. This material was then chromatographed (at 4°C) on a β3V anti-peptide antibody affinity column (12.5 cm x 0.8 cm), the column washed extensively with 0.1M Tris HCl pH 7.5, 10mM EGTA, 1mM PMSF, 1% Triton X-100, 1M NaCl until no protein could be detected in the eluate. TGF-β3 was then eluted in 50mM glycine, pH 2 into siliconized plastic vials. This material was pooled, acetonitrile was added to a final concentration of 25% and stored frozen at -20°C.

viii. C18 reverse phase was implemented at this stage prior to <u>in vivo</u> testing. The TGF-β3 eluted from the antibody affinity column was applied to a Waters C18 reverse phase HPLC column and developed using a 0-60% acetonitrile 0.1% TFA gradient, a flow rate 0.5 ml/minute and monitored using a 210nm flow through detector. Material was aliquoted and stored in the elution solvent at -20°C.

Quantitation of chr matographic yields was accomplished by Western bl t using β3V anti-peptide antibody r by antigen capture assay. The CCL-64 cell bioassay was utilized to test conditioned media and purified chromatographic fractions for biological activity of the recombinant TGF-β3 protein. The growth inhibition assay is based on measurement of DNA synthesis (18) using the decrease in 5'[^{125}I]-iodo-2'-deoxyuridine incorporation of treated compared to nontreated cells as a measure for growth inhibition. Conditioned media was acid activated and tested at several dilutions.

Fractions were analyzed by silver stain and Western blot and peak fractions pooled. A silver stained gel identified a single band of 12 kDa and 24 kDa under reducing and non-reducing conditions, respectively (Figure 20). The detection of a single silver staining band indicates that the preparation is greater than 95% homogeneous.

We further describe a novel purification protocol which allows the isolation of the glycosylated precursor form of TGF-β3 or the pro region of the TGF-β3 precursor using a lectin affinity column. An example of this is to use commercially available immobilized lectin such as wheat germ agglutinin bound to agarose (e.g. Sigma). The lectin column is washed and equilibrated with 5 column volumes of binding buffer (0.15 M NaCl 50 mM Hepes pH 7.6, 0.1% Triton X-100). Cell lysates or cell conditioned media containing the glycosylated precursor form of TGF-β3 is suspended in binding buffer and slowly loaded onto the lectin affinity column. It may be necessary to pass the buffer containing TGF-β3 up to 3 times to maximize binding of the protein to the column. The column is washed with 5 column volumes of the binding buffer to remove unbound material. The column is eluted with binding buffer containing 0.3 M N-acetylglucosamine to elute the bound glycosylated TGF-β3 precurs r pr t in from the column. Further, TGF-β3 is

separated from the other glycoproteins and treated to release the biologically active homodimer from the precursor complex. Other examples of lectins which could be used include but not be limited to ricin, abrin, and Con A.

The precursor form of TGF-β3 or the pro region of the TGF-β3 precursor may further purified using chromatographic steps known to those skilled in the art. For example, by sequential gel filtration chromatography, ion exchange chromatography, antibody-column chromatography, and high pressure liquid chromatography, or combinations thereof.

Mutants of the TGF-β3 precursor or mutants of mature TGF-β3 can be purified using the methods described for the TGF-β3 precursor and mature TGF-β3 as described herein.

EXAMPLE 5: ANTIBODIES WHICH NEUTRALISE TGF-β3 ACTIVITY

Human platelet TGF-β1 (Collaborative Research, MA), porcine TGF-β2 (R&D, Minnesota) or purified recombinant human TGF-β3, at concentrations from 3.125 to 0.049 ng/ml, was incubated with 5 μg/ml of affinity purified polyclonal rabbit antibodies (β3V antibody and anti-TGF-β (R&D, Minnesota) for 3 hours at 37°C. Control TGF-β3, TGF-β2 or TGF-β1 was incubated without antibodies. Growth inhibition of mink cells by antibody treated and control untreated TGF-β3, TGF-β2 or TGF-β1 was determined as described above. Figures 21A, 21B and 21C show that the β3V antibody (closed squares) neutralizes the growth inhibitory activity of TGF-β3, but not TGF-β2 or TGF-β1 on mink cells relative to the growth inhibitory activity of identical concentrations of TGF-β's in the absence of antibody (open circles). Anti-TGF-β (R&D, Minnesota) neutralizes TGF-β3, TGF-β2 and TGF-β1 (Figures 21A, 21B and 21C)(closed circles). Neither antibody had any significant effect on the growth of CCL-64 cells in the absence of TGF-β3. Antibodies

against the TGF-β3 peptide β3V apparently specifically neutralizes the growth inhibitory activity f TGF-β3.

EXAMPLE 6: BIOLOGICAL CHARACTERIZATION OF TGF-β3 IN VITRO

Growth was determined using a modification of the monolayer assay for TGF-β3 described by Iwata, K.K., et al. (19). Non-leukemic cells were subcultured on 96-well tissue culture plates in 100μl of media at a seeding density of $2x10^3$ cells per well. Cells were maintained and assayed in Dulbecco's modified Eagle's medium containing 10% fetal bovine and 2% L-glutamine. These cells were treated with 25 ng/ml (~1nM) of TGF-β3, pulsed 24 hours with 1μCi/ml 5-[^{125}I]-iodo-2'deoxyuridine when cells in the untreated control wells were 90% confluent and harvested.

Leukemic cells (K562, KG-1, KG-1a, HuT 78 and U937) were seeded in 50μl of media. K562 was seeded at a density of $1x10^3$ cells per well in RPMI supplemented with 10% fetal bovine serum. KG-1 and KG-1a were seeded at a density of $3.5x10^3$ cells per well in Iscove's media supplemented with 10% fetal bovine serum. Hut 78 and U937 were seeded at a density of $3.5x10^3$ cells per cell in RPMI supplemented with 10% fetal bovine serum. Cell growth was determined by microscopic examination. Examples are shown in Table 2, showing inhibition of some human tumor lines by TGF-β3.

EXAMPLE 7: DEVELOPMENT OF ANTIGEN CAPTURE ASSAY FOR TGF-β3

Plates are coated with 50 μl of affinity-purified rabbit polyclonal antibody (5μg/ml in 0.1M $NaHCO_3$, pH 9.1) made t TGF-β3 peptide β3V. Plates were incubated overnight at 4°C. Unbound antibody is removed by aspiration. Plates are blocked with 100 μl PBS containing 1% BSA (PBS-BSA) for 1 hr at room temperature. The plates are then washed twice with ph sphate-

buffer d salin (PBS) c ntaining 0.05% Tween 20 (PBST).

Samples in a final volume of 50 µl of PBS-BSA are added to the appropriate wells and incubated for 1 hr at room temperature. Unbound protein is removed and the plate is washed four times with PBST. All wells receive 50 µl of mouse monoclonal antibody against TGF-β3 (5µg/ml in PBS). After incubation for 1 hr at room temperature, unbound antibody is removed and the plate is washed four times with PBST. All wells receive 50 µl of an appropriate dilution of alkaline phosphatase conjugated to goat anti-mouse antibody. After incubation for 1 hr at room temperature, the plate was washed four times with PBST.

Substrate for alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate) in 100 µl is added to all of the wells and incubated for 15 min at room temperature. Absorbance in each well measured at 490 nm. Using this assay, we detected between 3-5ng/ml recombinant TGF-β3.

EXAMPLE 8: EXPRESSION OF CELLULAR AND MEMBRANE-ANCHORED TGF-β3

Expression of biologically active and inactive TGF-β3 may be achieved in eukaryotic cells such that the final protein product is retained by the cell and not released into the cell culture media. This has advantages over release of TGF-β3 into media in concentrating TGF-β3 to the cell membrane during recovery. Protein sorting of TGF-β3 within a cell to the endoplasmic reticulum (ER), the plasma membrane or extracellular matrix is achieved through incorporation of specific targeting signals into the TGF-β3 precursor or mature TGF-β3 such that targeting signal sequences can be removed

TABLE 2

Effects of TGF-β3 (1nM) on the Growth of Human Cell Lines in Culture

CELL LINE		% INHIBITION
Human Tumor		
A549	(lung adenocarcinoma)	46
A375	(melanoma)	47
A2058	(melanoma)	88
WiDR	(colon adenocarcinoma)	24
MCF 7	(breast carcinoma)	57
Human Leukemic Cells		
K562	(CML)	55
KG-1	(AML)	50
KG-1a	(AML)	50
HuT 78	(T cell lymphoma)	50
U937	(histiocytic lymphoma)	50
Normal Human		
Huf	(foreskin fibroblasts)	6

during recovery and purification. For example, TGF-β3 cDNA could be modified so as to produce a TGF-β3 precursor protein which is membrane anchored via a hydrophobic transmembran sequence. Briefly, such TGF-β3 proteins are expressed as follows.

The molecular biology techniques use standard methods (23). High level expression vectors for production of TGF-β3 are constructed with the following properties. Transcription of TGF-β3 derives from a strong promoter coupled with the enhancers/replication origins for SV40 virus and polyoma virus to further enhance transcription and allow replication in COS cells or polyoma transformed cell lines for short term analysis of the constructs. Expression vectors additionally contain sequences stabilizing the RNA and increasing its half-life (e.g. untranslated sequences from the rabbit β-globin 5'and bovine growth hormone 3' regions), and contain splicing signals to additionally stabilize the RNA transcript (immunoglobulin intron sequences or SV40 small t intron sequences). The vectors contain a Kozak consensus sequence surrounding the initiating methionine to promote efficient translation of the mRNA. TGF-β2 and TGF-β3 lack a consensus signal peptide cleavage sequence as defined by Blobel (a basic amino acid, preferably Lys or Arg, followed by an amino acid with a small side chain, commonly Gly or Ala). The TGF-β3 preproprecursor is processed to the precursor form by cleavage of a 22-24 amino acid hydrophobic signal peptide commitant to translocation across the endoplasmic reticulum presumably at a cryptic cleavage site. This site can be mutated to a consensus Arg-Ala cleavage sequence using standard site specific mutagenesis techniques to promote optimal secretion/translocation or deleted to promote cytoplasmic localization.

At the carboxyl-terminus of the precursor protein, DNA encoding a hydrophobic transmembrane amino acid sequence (e.g. that encoded by the epidermal growth factor receptor cDNA or c-erbB2 cDNA) followed by a highly charged 'stop transfer' amino acid sequence is inserted such that a TGF-β3 precursor protein modified in this way binds to the plasma membrane. An amino acid recognition site for a specific protease (e.g. factor X or collagenase) is included between the C-terminal transmembrane sequence and the N-terminal TGF-β3 precursor to allow efficient cleavage of the TGF-β3 from the membrane by specific proteases (e.g. factor Xa or collagenase) (Figure 22). An example of this procedure is as follows:

1. A mutation oligonucleotide [5' CTCTGTCGCACGTGGATCCTCAGCTA 3'] is used to engineer a BamHI restriction endonuclease cleavage site 3' of the TGF-β3 termination codon.

2. A second mutation oligonucleotide is used by one skilled in the art to remove the TGF-β3 termination codon.

3. Synthetic oligonucleotides are constructed to introduce the protease cleavage site (for example Factor Xa: IleGluGlyArg or collagenase: Pro-X-Gly-Pro) followed by the sequence encoding the c-erbB2 transmembrane sequence and stop transfer sequences (ThrSerIleValSerAlaValValGlyIleLeuLeuValValValLeuGly ValValPheGlyIleLeuIleLysArgArgGlnGlnLysIleArgLysTyrThr Met) such that sequences are inserted in the same translated reading frame as the TGF-β3 precursor. The nucleic acid molecule produced is inserted in an expression vector and introduced into a host cell line (eg., CHO and HeLa cells).

Alternatively, linkage of the transmembrane and cytoplasmic domains of vesicular stomatius virus glycoprotein to the C-

t rminus of the TGF-β3 precursor with an intervening pr tease cleavage s qu nce (e.g. Factor Xa or collag nas) allows for the production of membrane anchored TGF-β3 precursor, as described for rat growth hormone (16).

A number of proteins are anchored to the cell membrane via covalent phosphatidyl inositol linkage (e.g. Qa-2, decay accelerating factor (DAF), Thy-1) (21). Expression of membrane anchored TGF-β3 may also be achieved by inclusion of phosphatidyl inositol linkage sequences. It is preferable, though not required, that the phosphotidyl inositol linked TGF-β3 precursor protein may be freed from the membrane by treatment with phospholipase C.

For example, inclusion of the C-terminal 37 amino acids of DAF (PNKGSGTTSGTTRLLSGHTCFTLTGLLGTLVTMGLLT) (4) linked by a Factor Xa protease cleavage sequence (I-D-G-R) or collagenase cleavage sequence (P-X-G-P) to the TGF-β3 precursor sequence would link TGF-β3 to the cell in which it is produced by a glycophospholipid membrane anchor attached to the DAF sequence.

The C-terminal sequences of variant surface glycoprotein (VSG) of Trypanosoma brucei have been shown to be linked to the plasma membrane by a phospholipase C sensitive anchor (21). Linkage of this sequence to C-terminus of the TGF-β3 precursor via a protease cleavage sequence as previously described enables the synthesis of membrane bound TGF-β3 precursor protein.

Alternatively, TGF-β3 precursor protein may be targeted to the endoplasmic reticulum by deletion of TGF-β3 signal peptide sequences such that the resulting protein begins with leucine at nucleotide positions 332-334 and by inclusi n of an endoplasmic reticulum targeting sequence, for example, the

r tavirus SAII glyc protein VP7 signal sequence (36) int the TGF-β3 pr curs r sequence. Alternativ ly, the VP7 signal sequence may be linked to the C-terminus of the mature TGF-β3 such that a protease cleavage sequence (e.g. Factor Xa or collagenase) separates the mature TGF-β3 from the VP7 signal sequence.

EXAMPLE 9: OPTIMISATION OF EXPRESSION AND BIOLOGICAL ACTIVITY: STRUCTURE FUNCTION RELATIONSHIPS

TGF-βs exert their biological effects through receptor binding. Three putative receptors have been identified by chemical cross-linking, and are designated type I, type II, and type III receptors with molecular weights 65 kD, 85 kD and ~280 kD, respectively (5, 6). The biological effects of TGF-β1, -2, and -3 appear to be mediated by type I and/or type II receptors. Cells which lack the type III receptor still respond to TGF-βs; thus, type III receptor binding does not appear essential for at least many of the biological activities of the TGF-βs.

Type III receptor is present at high concentration in the body, relative to type I and II receptors. Type III receptors exists in two forms, a membrane proteoglycan which binds TGF-β via the N-glycosylated core protein and betaglycan, a secreted, soluble form associated with the extracellular matrix (1). Accordingly, it may be possible to mutate TGF-β3 to prevent binding specifically to the type III receptors, thereby increasing the half life <u>in vivo</u> of retained activity.

TGF-βs are also known to bind serum proteins, such as α_2 macroglobulin (29, 18). Thus, binding of TGF-β3 to α_2 macrogl bulin r t other serum pr teins may als sequester,

inactivat or pr mote clearanc f TGF-β3. TGF-βs are also known t bind the TGF-β pro region and are inactivated in that complex. TGF-βs have a short half-life in vivo (estimated at 2.2 minutes) (7).

Degenerate Oligonucleotide Directed Mutagenesis

In order to investigate binding of TGF-β3 to proteins involved in clearance or inactivation the regions of TGF-β3 that interact with these proteins (such as α_2 macroglobulin and the secreted form of the betaglycan receptor of TGF-β) are subjected to mutagenesis using degenerate oligonucleotides (5, 25) and, subsequently, recombinant mutant TGF-β3 are tested for their affinity for such proteins.

The degenerate oligonucleotides spanning the regions involved with binding or clearance proteins are designed such that the 3' ends contain an 8 nucleotide palindromic sequence encompassing a restriction endonuclease cleavage site. Preferably, the 5' end of the oligonucleotide consists of sequences encompassing another restriction endonuclease site. The central region (20-100 bp) contains the mutagenized sequence of the TGF-β3 interaction site. In order to introduce point mutations in the desired regions, the synthesis of the oligonucleotides are programmed such that positions where mutation are not desired (e.g., the restriction sites) are synthesized using solutions of the individual phosphoramidites, while the regions where mutations are desired are synthesized using a defined mixture of phosphoramidites. Using this procedure, a 10% mutation rate can be achieved by a mixture of 90% wild type nucleotide and 3.3% of each of the three "incorrect" nucleotides. Following synthesis, the oligonucleotides are purified either with Sep Pak C18 cartridge for sh rt ligonucle tides (<50 nt) r by d naturing preparativ polyacrylmide electroph resis for

longer oligonucleotides.

The conversion to double-stranded DNA is achieved by self annealing of the oligonucleotides using the 3' (and 5') end palindromic sequences and treatment with DNA polymerase Klenow fragment in the presence of the four dNTPs. After digestion with the restriction enzymes, recognizing the 5' and 3' outside sites, the double stranded oligonucleotide mixture is purified by non-denaturing polyacrylamide gels. Th oligonucleotide mixture is then be used to replace the corresponding fragment in the TGF-β3 expression plasmid. A double strand sequencing method is used to directly determine the genotype of the mutants.

If the mutation frequency is too low, the mutant clones are identified by differential colony filter hybridization using the kinased 5' ^{32}P labeled oligonucleotide as a probe. The TGF-β3 mutant expression constructs are transfected into COS cells. Subsequently, the TGF-β3 protein is partially purified using a TGF-β3 immunoaffinity column and tested for ability to interact with TGF-β3 binding/inactivating proteins.

Saturation Mutagenesis

In order to create a large number of randomly distributed nucleotide substitutions in the cDNA fragment encoding the complete mature TGF-β3, a saturation mutagenesis method is applied. The resulting TGF-β3 mutants are tested for biological activity, receptor binding capacity and affinity for TGF-β binding proteins. Finally, the in vivo half-life of a selected group of mutants is tested.

First the Bluescript (Stratagene) plasmid containing the TGF-β3 cDNA is subjected to site directed mutagenesis in order to create a FspI site at R-K-K-R processing site of TGF-β3

(preceding the matur pr tein) and a BamHI site 3' of TGF-β3 stop cod n. The foll wing mutation olig nucleotid s will be used:

5' GTAATTGGTGTCCAATGCGCACTTCTTCCTCTG 3' and

FspI

5' CTCTGTCGCACGTGGATCCTCAGCTA 3'

BamHI

These sites facilitate removal from and reinsertion of the mutant fragment into the TGF-β3 cDNA plasmid. The FspI - BamHI TGF-β3 cDNA target fragment is subcloned in M13 into both orientations relative to a M13 origin of replication.

Three different reactions are performed on each of the strands of the mutagenized target sequence (40 µg of 1 mg/ml single stranded DNA per tube).

1. 10 µl of Sodium acetate, pH 4.3 and 50 µl 2 M sodium nitrite is added and incubated for 60 min at room temperature.

2. 60 µl concentrated (18 M) formic acid is added and incubated for 10 min room temperature.

3. 60 µl of concentrated (12 M) hydrazine is added and incubated for 10 min at room temperature.

After incubation, DNA's are ethanol precipitated, washed with 70% ethanol and resuspended in TE buffer. The 2nd strand are prepared by primer extensi n using AMV r vers transcriptase (depurinati n inhibits DNA synthesis by E.coli DNA pol I but

n t by reverse transcriptase). Mutants can be identified directly by DNA sequencing f rand m clones.

Binding of TGF-β3 to α_2 macroglobulin

The binding of α_2-macroglobulin to TGF-β3, modified TGF-β3 or TGF-β3 bound to anti-TGF-β3 antibody is measured using a modification of the method described by O'Conner-McCourt, et al. (29). Briefly, ^{125}I TGF-β3 is incubated with α_2-macroglobulin in PBS with either unlabeled TGF-β3, modified TGF-β3 or TGF-β3 bound to anti-TGF-β3 antibody for 5 hr on ice. Non-specific binding will be determined using a 400-fold molar excess of unlabeled growth factor. Crosslinking of th α_2-macroglobulin to the ^{125}I TGF-β3 is accomplished with the addition of a ¼ volume of 5 mM bis(sulfosuccinimidyl)suberat (BS^3; Pierce) in PBS and the reaction is stopped after 2 min at 4°C by the addition of 1/20 volume of 2.5 M glycine. An equal volume of SDS-PAGE sample buffer (2X) is added to the sample will be heated in a boiling water bath for 3 min.

Electrophoresis is performed on the samples and destained gels is dried and exposed at -70°C to X-ray film using intensifying screens. Alternatively, commercially available anti-α_2-macroglobulin (Sigma) is used to immunoprecipitate the α_2-macroglobulin ^{125}I TGF-β complex with or without crosslinking and quantitated directly by a gamma counter.

Binding assay of TGF-β3 to type III receptors

Type III receptors exist in both a membrane bound (proteoglycan) and soluble (betaglycan) forms. The binding of betaglycan to TGF-β, modified TGF-β or TGF-β bound to anti-TGF-β antibody is measured using the method described by Andres, et al (1). Non-specific binding is determined using a 400-f ld m lar excess of unlabeled growth factor. Briefly,

soluble betaglycan is made from serum-free conditioned media from 3T3-L1 adipocytes. Mouse 3T3-L1 adipocytes is grown to confluence or near confluency. The cell monolayers are washed twice and incubated for 3 days in serum-free Waymouth's medium. A concentrated stock of fresh PMSF is added to a final concentration of 0.2 mM immediately after collection. The collected conditioned media is centrifuged at 2,000 x g for 15 min at 4°C followed by ultracentrifugation at 2000,000 x g for 40 min at 4°C. NaCl is added to the conditioned media to a final concentration of 0.25 m NaCl and brought to pH 6.0 by the addition of 1 M BIS-Tris, pH 6.0 and pre-equilibrated with binding buffer (50 mM NaCl, 10 mM $MgCl_2$, 5 mM KCI, 25 mM Hepes, pH 7.5 containing 1 mg/ml of BSA). ^{125}I TGF-β is mixed with incubated with unlabeled TGF-β, modified TGF-β or TGF-β bound to anti-TGF-β antibody and soluble betaglycan (appropriately diluted) in binding buffer. This mixture is added to the pre-equilibrated DEAE-Sepharose Fast Flow and incubated for 3.5 hours at 4°C with continued mixing. The beads are then washed 5 times with cold binding buffer (without albumin). The amount of radioactivity remaining on the beads will be quantitated. For analysis of specific binding to the type III proteoglycan receptors, ^{125}I TGF-β3 is cross-linked to intact cells (6), the receptors separated by standard SDS-PAGE, and ^{125}I-binding quantitated by densitometry.

Pharmacokinetics of TGF-β3

The chemical half-life of TGF-β3 is determined in the serum of mice following bolus injections (0.1-10μg/mouse) via i.v., i.p., and s.c. routes, using internally labelled TGF-β3 (labelled metabolically with ^{35}S cysteine) or ^{125}I TGF-β3. Tissue distribution of labelled material is measured in various organs with particular emphasis on liver, spleen and bone marrow sites. If the biological half-life of TGF-β3 in

vivo is found to be unacceptably short regional administration by direct intrasplenic injection (through the body wall) is employed or using the surgical technique reported by Goey et al (12) involving injection into the femoral artery. This latter approach has been reported to be effective in localizing TGF-β1 to the marrow with resulting inhibition of early stem cell and progenitor cell proliferation.

Example 10: Expression of a Pro Region of the TGF-β3 Precursor

The TGF-β3 pro region protein associates with the mature TGF-β3 and modifies the half life and biological activity of mature TGF-β3. Nucleic acid encoding the TGF-β3 precursor beginning with methionine at nucleotide positions 263-265 and ending with arginine at position 1160-1162 is engineered by mutagenesis of the nucleic acid in Figure 1 to introduce a translation termination codon (TGA, TAG, TAA) at position 1163-1165. The resulting nucleic acid is inserted in an expression vector and transfected into a suitable host cell with an additional selectible marker, as previously described. TGF-β3 pro region protein is recovered from the culture medium. This protein stably binds mature TGF-β3 and thereby sequesters and modifies the half life and biological activity of the mature TGF-β3.

Binding assay of TGF-β3 to the TGF-β3 pro region protein

The binding of TGF-β3 pro region to TGF-β3 or mutant TGF-β3 is measured by the following. ^{125}I TGF-β3 is incubated with purified TGF-β3 pro region in PBS with either unlabeled TGF-β3 or mutant TGF-β3 for 5 hr on ice. Non-specific binding will be determined using a 400-fold molar excess of unlabeled growth factor. Crosslinking of the TGF-β3 pro region to ^{125}I TGF-β3 is accomplished with the addition of a ¼ volume of 5 mM bis(sulfosuccinimidyl)suberate (BS^3; Pierce) in PBS and the

reacti n is stopped after 2 min at 4°C by the additi n of 1/20 volume of 2.5 M glycin . An equal volum f SDS-PAGE sample buffer (2X) is added to the sample will be heated in a boiling water bath for 3 min.

Electrophoresis is performed on the samples and destained gels is dried and exposed at -70°C to X-ray film using intensifying screens. Alternatively, antI-TGF-ß3 is used to immunoprecipitate the TGF-ß3 pro region complexed with ^{125}I TGF-β complex with or without crosslinking and quantitated directly by a gamma counter.

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37. Wrann, M. et al. (1987) EMBO J. 6:1633-1636.

38. U.S. Patent No. 4,886,747, issued December 12, 1989 to R. Derynck and D. Goeddell.

What is claimed is:

1. A method for recovering purified, non-denatured mature TGF-β3 from a mixture of mammalian cell-derived polypeptides which comprises contacting the mixture with an antibody which specifically binds to mature TGF-β3 but exhibits substantially no cross reactivity with mature TGF-β1 and mature TGF-β2.

2. The method of claim 1, wherein the mixture of mammalian cell-derived polypeptides includes a mixture of non-human mammalian polypeptides from non-human cells in which TGF-β3 has been expressed.

3. The method of claim 1, wherein the antibody is directed to an epitope defined by the amino acid sequence YLRSADTTHSTVLGLYNTLNPEASASY.

4. The method of claim 3, wherein the antibody is immobilized on a solid support.

5. A method for producing substantially purified TGF-β3 precursor having an anchorage membrane sequence which comprises:

(a) preparing DNA encoding a TGF-β3 precursor having the membrane anchorage sequence;

(b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF-β3 precursor is expressed and subsequent translocation of the expressed TGF-β3 precursor having the membrane anch rage sequenc ;

(d) culturing the host cell in medium;

(e) separating the host cell from the medium;

(f) disrupting the h st cell such that a lysate containing the TGF-β3 precursor having the membrane anchorage sequence is produced; and

(g) purifying the TGF-β3 precursor having a membran anchorage sequence from the lysate under conditions such that the substantially purified TGF-β3 precursor is produced.

6. A method for producing substantially purified TGF-β3 precursor having an anchorage membrane sequence which comprises:

(a) preparing DNA encoding the TGF-β3 precursor having a membrane anchorage sequence;

(b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF-β3 precursor is expressed and subsequent occlusion of the expressed TGF-β3 precursor having a membrane anchorage sequence in occlusion bodies;

(d) culturing the host cell in culture medium;

(e) separating the occlusion bodies from the host cells and the culture medium;

(f) disrupting the occlusion bodies to produce a solution containing the TGF-β3 precursor having a membrane anchorage sequence; and

(g) purifying the TGF-β3 precursor having a membrane anchorage sequence from the lysate such that the substantially purified TGF-β3 precursor is produced.

7. The method of any of claims 5 or 6, wherein the anchorage linkage sequence includes a phosphatidyl inositol linkage.

8. The method of any of claims 5 or 6, wh rein the anchorage linkage sequence includes a hydrophobic transmembrane peptide sequence.

9. A method for producing a substantially purified mutant TGF-β3 precursor which comprises:

(a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498;

(b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF-β3 precursor is expressed;

(d) culturing the host cell in a medium under conditions such that the expressed mutant TGF-β3 precursor is secreted into the medium;

(e) separating the cell from the medium containing the mutant TGF-β3 precursor so secreted; and

(f) purifying the mutant TGF-β3 precursor thereby producing the substantially purified mutant TGF-β3 precursor.

10. A method for producing a substantially purified mutant TGF-β3 precursor which comprises:

(a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamin encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498;

(b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF-β3 precursor is expressed;

(d) separating the host cell containing the expressed, mutant TGF-β3 precursor from the culture medium;

(e) disrupting the host cell such that a lysate containing the mutant TGF-β3 precursor is produced; and

(f) purifying the mutant TGF-β3 precursor thereby producing the substantially purified mutant TGF-β3 precursor.

11. A method for producing a substantially purified mutant TGF-β3 precursor which comprises:

(a) preparing a DNA c mprising a first DNA sequence

encoding an amin acid sequence substantially id ntical to the amino acid sequ nce shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked t nucleotide 1150, a third DNA sequence, linked t the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498;

(b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a mutant TGF-β3 precursor is expressed;

(d) culturing the host cell in medium under conditions such that the expressed mutant TGF-β3 precursor is secreted into the medium;

(e) separating the cell from the culture medium containing the mutant TGF-β3 precursor so secreted; and

(f) purifying the mutant TGF-β3 precurs r such that a substantially purified mutant TGF-β3 precurs r is

produced.

12. A method for producing a substantially purified mutant TGF-β3 precursor which comprises:

(a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked t nucleotide 1150, a third DNA sequence, linked t the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498;

(b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a mutant TGF-β3 precursor is expressed;

(d) separating the host cell containing the expressed, mutant TGF-β3 precurs r from the culture medium;

( ) disrupting the host cell such that a lysate

containing the mutant TGF-β3 precurs r is produced; and

(f) purifying the mutant TGF-β3 precursor such that a substantially purified mutant TGF-β3 precursor is produced.

13. The method of any of claims 11 or 12, wherein in step (a) X is selected from a group of tri-nucleotides consisting of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT, TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT, CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, ATA, ACT, ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG, GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA, GAG, GGT, GGC, GGA, and GGG.

14. The method of any of claims 5, 6, 9, 10, 11, or 12, wherein purification includes affinity chromatography.

15. The method of any of claims 5, 6, 9, 10, 11, or 12, wherein affinity chromatography comprises lectin column chromatography.

16. The method of claim 14, wherein affinity chromatography comprises antibody column chromatography.

17. The method of any of claims 9 or 10, wherein in step (a) the protease cleavage sequence includes a collagenase recognition sequence.

18. The method of any of claims 9 or 10, wherein in step (a) the protease cleavage sequence includes a Factor Xa recognition sequence.

19. The m thod f any f claims 5 or 6 which further comprises:

(a) treating the purified TGF-β3 precurs r s rec vered with an activating agent to separate a mature TGF-β3 from the precursor; and

(b) recovering the separated mature TGF-β3 of step (a).

20. The method of any of claims 9 or 10 which further comprises:

(a) treating the purified mutant TGF-β3 precursor so recovered with an activating agent to separate a mature TGF-β3 from the precursor; and

(b) recovering the separated mature TGF-β3 of step (a).

21. The method of any of claims 11 or 12 which further comprises:

(a) treating the purified mutant TGF-β3 precursor s recovered with cyanogen bromide to separate a mature TGF-β3 from the precursor; and

(b) recovering the separated mature TGF-β3 of step (a).

22. A method for producing mutant TGF-β3 which comprises:

(a) preparing a DNA encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a leucine encoded by nucleotides 332-334 and ending with a serine encoded by nucleotides 1496-1498;

(b) inserting the DNA of step (a) into an expression vector operably linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a mutant TGF-β3 is expressed;

(d) culturing the host cell in medium;

(e) separating the host cell containing the mutant TGF-

ß3 s xpr ssed fr m the m dium;

(f) disrupting th cell to produce a lysate c ntaining the mutant TGF-ß3; and

(g) purifying the mutant TGF-ß3.

23. The method of claim 22 which further comprises:

(a) treating the purified mutant TGF-ß3 so recovered with an activating agent to separate a mature TGF-ß3 from the mutant TGF-ß3; and

(b) recovering the separated mature TGF-ß3 of step (a).

24. A process which comprises:

(a) contacting a TGF-ß3 precursor with a precipitating agent thereby concentrating the TGF-ß3 precursor in a precipitate;

(b) extracting the pellet of step (a) with an acidified organic solution under such conditions that mature TGF-ß3 is separated from the pellet; and

(c) recovering the mature TGF-ß3 so separated in step (b).

25. The process of claim 24, wherein the acidified organic solution in step (b) includes acidified acetonitrile.

26. The process of claim 25, wherein the acidified acetonitrile comprises 50% acetonitrile and 1.0M acetic acid.

27. The process of claim 24, wherein the precipitating agent in step (a) includes ammonium sulfate.

28. A method for producing and identifying a mutant, mature TGF-ß3 exhibiting reduced binding affinity to serum binding proteins which c mpris s:

(a) preparing a DNA encoding the TGF-ß3;

(b) performing mutagenesis n the DNA f step (a) thereby btaining a mutant DNA;

(c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell;

(d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF-β3 is expressed;

(e) culturing the host cell in medium under conditions such that the expressed mutant TGF-β3 is secreted into the medium;

(f) separating the host cell from the culture medium containing the mutant TGF-β3 so expressed;

(g) purifying the mutant TGF-β3;

(h) activating the mutant TGF-β3 so expressed under conditions such that a mutant mature TGF-β3 is separated from the mutant TGF-β3; and

(i) assaying the culture medium for the mutant, mature TGF-β3 thereby identifying a mutant, mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins.

29. A method for producing and identifying a mutant, mature TGF-β3 exhibiting reduced binding affinity to serum binding proteins which comprises:

(a) preparing a DNA encoding the TGF-β3;

(b) performing mutagenesis on the DNA of step (a) thereby obtaining a mutant DNA;

(c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell;

(d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under c nditi ns such that a mutant TGF-β3 is

express d;

( ) culturing the host cell in m dium under conditi ns such that the expressed mutant TGF-ß3 is produced in the host cell;

(f) separating the host cell containing the mutant TGF-ß3 so expressed from the culture medium;

(g) disrupting the cells to produce a lysate containing the mutant TGF-ß3;

(h) purifying the mutant TGF-ß3;

(i) activating the mutant TGF-ß3 so expressed under conditions such that a mature TGF-ß3 is separated from the mutant TGF-ß3; and

(j) assaying the culture medium for the mutant, matur TGF-ß3 exhibiting reduced binding affinity to serum binding proteins thereby identifying a mutant, mature TGF-ß3 exhibiting reduced binding affinity to serum binding proteins.

30. The method of any of claims 28 or 29, wherein the serum binding protein is α2-macroglobulin.

31. The method of any of claims 28 or 29, wherein the serum binding protein is type III TGF-ß receptor.

32. The method of claim 31, wherein the type III TGF-ß receptor is betaglycan.

33. The method of any of claims 28 or 29, wherein the serum binding protein is a pro region of the TGF-ß precursor.

34. A method for producing a substantially purified pro region of the TGF-ß3 precursor which comprises:

(a) preparing DNA encoding the pro region of the TGF-ß3 precursor;

(b) inserting th DNA int an expr ssi n vector linked

to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF-β3 precursor is expressed;

(d) culturing the host cell in medium;

(e) separating host cells from the medium;

(f) disrupting the host cells to produce a lysate containing the pro region of the TGF-β3 precursor so expressed; and

(g) purifying the pro region of the TGF-β3 precursor from the lysate such that the substantially purified pro region of the TGF-β3 precursor is produced.

35. A method for producing a substantially purified pro region of the TGF-β3 precursor which comprises:

(a) preparing DNA encoding the pro region of the TGF-β3 precursor;

(b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;

(c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF-β3 precursor is expressed;

(d) culturing the host cell in medium under conditions such that the expressed pro region of the TGF-β3 precursor is secreted into the medium;

(e) separating host cells from the medium containing the pro region of the TGF-β3 precursor so secreted; and

(f) purifying the pro region of the TGF-β3 precursor such that the substantially purified pro region of the TGF-β3 precursor is produced.

36. Th method of any f claims 5, 6, 9, 10, 11, 12, 22, 28, 29, 34, r 35, wh rein the host c ll is a eucaryotic cell.

37. The method of any of claims 5, 6, 9, 10, 11, 12, 22, 28, 29, 34, or 35 wherein the host cell is a procaryotic cell.

FIGURE 1

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CCAGCAAAACCTGTTTAGACACATGGACAAGAATCCCAGCGCTACAAGGCACACAGTCCGCTTCTTCGTCCTCAGGGTTGCCAGCGCTTCCTGGAAGTCC
        10        20        30        40        50        60        70        80        90       100
TGAAGCTCTCGCAGTGCAGTGAGTTCATGCACCTTCTTGCCAAGCCTCAGTCTTTGGGATCTGGGGAGGCCGCCTGGTTTTCCTCCCTCCTTCTGCACGT
       110       120       130       140       150       160       170       180       190       200
                                                  Alu I↓        M  K  M  H  L  Q  R  A  L  V  V  L  A
CTGCTGGGGTCTCTTCCTCTCCAGGCCTTGCCGTCCCCCTGGCCTCTCTTCCCAGCTCACACATGAAGATGCACTTGCAAAGGGCTCTGGTGGTCCTGGC
       210       220       230       240       250       260       270       280       290       300
 L  L  N  F  A  T  V  S  L  S  L  S  T  C  T  T  L  D  F  G  H  I  K  K  K  R  V  E  A  I  R  G  Q
CCTGCTGAACTTTGCCACGGTCAGCCTCTCTCTGTCCACTTGCACCACCTTGGACTTCGGCCACATCAAGAAGAAGAGGGTGGAAGCCATTAGGGGACAG
       310       320       330       340       350       360       370       380       390       400
I  L  S  K  L  R  L  T  S  P  P  E  P  T  V  M  T  H  V  P  Y  Q  V  L  A  L  Y  N  S  T  R  E  L
ATCTTGAGCAAGCTCAGGCTCACCAGCCCCCCTGAGCCAACGGTGATGACCCACGTCCCCTATCAGGTCCTGGCCCTTTACAACAGCACCCGGGAGCTGC
       410       420       430       440       450       460       470       480       490       500
L  E  E  M  H  G  E  R  E  E  G  C  T  Q  E  N  T  E  S  E  Y  Y  A  K  E  I  H  K  F  D  M  I  Q  G
TGGAGGAGATGCATGGGGAGAGGGAGGAAGGCTGCACCCAGGAAAACACCGAGTCGGAATACTATGCCAAAGAAATCCATAAATTCGACATGATCCAGGG
       510       520       530       540       550       560       570       580       590       600
 L  A  E  H  N  E  L  A  V  C  P  K  G  I  T  S  K  V  F  R  F  N  V  S  S  V  E  K  N  R  T  N  L
GCTGGCGGAGCACAACGAACTGGCTGTCTGCCCTAAAGGAATTACCTCCAAGGTTTTCCGCTTCAATGTGTCCTCAGTGGAGAAAAATAGAACCAACCTA
       610       620       630       640       650       660       670       680       690       700
F  R  A  E  F  R  V  L  R  V  P  N  P  S  S  K  R  N  E  Q  R  I  E  L  F  Q  I  L  R  P  D  E  H
TTCCGAGCAGAATTCCGGGTCTTGCGGGTGCCCAACCCCAGCTCTAAGCGGAATGAGCAGAGGATCGAGCTCTTCCAGATCCTTCGGCCAGATGAGCACA
       710       720       730       740       750       760       770       780       790       800
I  A  K  Q  R  Y  I  G  G  K  N  L  P  T  R  G  T  A  E  W  L  S  F  D  V  T  D  T  V  R  E  W  L  L
TTGCCAAACAGCGCTATATCGGTGGCAAGAATCTGCCCACACGGGGCACTGCCGAGTGGCTGTCCTTTGATGTCACTGACACTGTGCGTGAGTGGCTGTT
       810       820       830       840       850       860       870       880       890       900
 R  R  E  S  N  L  G  L  E  I  S  I  H  C  P  C  H  T  F  Q  P  N  G  D  I  L  E  N  I  H  E  V  M
GAGAAGAGAGTCCAACTTAGGTCTAGAAATCAGCATTCACTGTCCATGTCACACCTTTCAGCCCAATGGAGATATCCTGGAAAACATTCACGAGGTGATG
       910       920       930       940       950       960       970       980       990      1000
E  I  K  F  K  G  V  D  N  E  D  D  H  G  R  G  D  L  G  R  L  K  K  Q  K  D  H  H  N  P  H  L  I
GAAATCAAATTCAAAGGCGTGGACAATGAGGATGACCATGGCCGTGGAGATCTGGGGCGCCTCAAGAAGCAGAAGGATCACCACAACCCTCATCTAATCC
      1010      1020      1030      1040      1050      1060 *    1070      1080      1090      1100
L  M  M  I  P  P  H  R  L  D  N  P  G  Q  G  G  Q  R  K  K  R  A  L  D  T  N  Y  C  F  R  N  L  E  E
TCATGATGATTCCCCCACACCGGCTCGACAACCCGGGCCAGGGGGGTCAGAGGAAGAAGCGGGCTTTGGACACCAATTACTGCTTCCGCAACTTGGAGGA
      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
 N  C  C  V  R  P  L  Y  I  D  F  R  Q  D  L  G  W  K  W  V  H  E  P  K  G  Y  Y  A  N  F  C  S  G
GAACTGCTGTGTGCGCCCCCTCTACATTGACTTCCGACAGGATCTGGGCTGGAAGTGGGTCCATGAACCTAAGGGCTACTATGCCAACTTCTGCTCAGGC
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
```

FIGURE 1

-continued-

```
P  C  P  Y  L  R  S  A  D  T  T  H  S  T  V  L  G  L  Y  N  T  L  N  P  E  A  S  A  S  P  C  C  V
CCTTGCCCATACCTCCGGCAGTGCAGACACAACCCACAGCACGGTGCTGGGACTGTACAACACTCTGAACCCTGAAGCATCTGCCTCGCCTTGCTGCGTGC
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
P  Q  Q  L  E  P  L  T  I  L  Y  Y  Y  V  G  R  T  P  K  V  E  Q  L  S  N  M  V  V  K  S  C  K  C  S
CCCAGGACCTGGAGCCCCTGACCATCCTGTACTATGTTGGGAGGACCCCCAAAGTGGAGCAGCTCTCCAACATGGTGGTGAAGTCTTGTAAATGTAGCTG
      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
AGACCCCACGTGCGACAGAGAGAGGGGAGAGAACCACCACTGCCCGCTCCTCGGGAAACACACAAGCAACAAACCTCACTGAGAGGCCTG
      1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
GAGCCCACAACCTTCGGCTCCGGGCAAATGGCTGAGATGGAGGTTTCCTTTTGGAACATTTCTTTCTTGCTGGCTCTGAGAATCACGGTGGTAAAGAAAG
      1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
TGTGGGTTTGGTTAGAGGAAGGCTGAACTCTTCAGAACACACAGACTTTCTGTGACGCAGACAGAGGGGATGGGGATAGAGGAAAGGGATGGTAAGTTGA
      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
GATGTTGTGTGGCAATGGGATTTGGGCTACCCTAAAGGGAGAAGGAAGGGCAGAGAATGGCTGGGTCAGGGCCAGACTGGAAGACACTTCAGATCTGAGG
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
TTGGATTTGCTCATTGCTGTACCACATCTGCTCTAGGGAATCTGGATTATGTTATACAAGGCAAGCATTTTTTTTTTTTTTTAAAGACAGGTTACGAAGA
      1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
CAAAGTCCCAGAATTGTATCTCATACTGTCTGGGATTAAGGGCAAATCTATTACTTTGCAAACTGTCCTCTACATCAATTAACATCGTGGGTCACTACA
      2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
GGGAGAAAATCCAGGTCATGCAGTTCCTGGCCCATCAACTGTATTGGGCCTTTTGGATATGCTGAACGCAGAAGAAAGGGTGGAAATCAACCCTCCTCTG
      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
TCTGCCTCTGGGTCCCTCCTCTCACCTCTCCCTCGATCATATTTCCCCTTGGACACTTGGTTAGACGCCTTCCAGGTCAGGATGCACATTTCTGGATTGT
      2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
GGTTCCATGCAGGGTTGGGGCATTATGGGTTCTTCCCCCACTTCCCCTCCAAGACCCTGTGTTCATTTGGTGTTCCTGGAAGCAGGTGCGACAACATGTG
      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
AGGCATTCGGGGAAGCTCGACATGTGCCACACAGTGACTGAGGTATAAAGACAAGTATGAATATTACTCTCAAAATCTTT
      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
GTATAAATAAATATTTTTGGGGCATCCTG poly(A)
      2510      2520
```

FIGURE 2

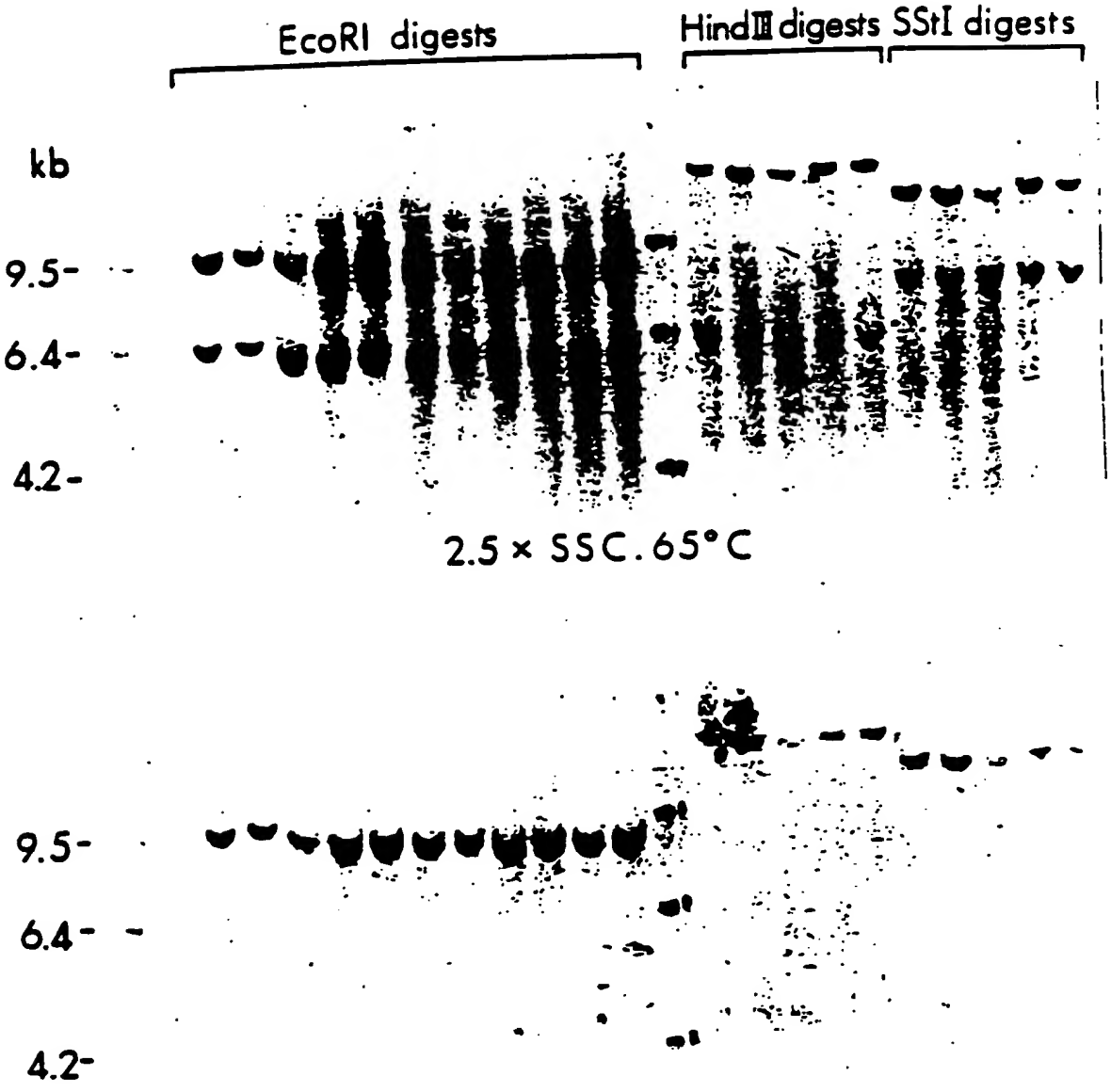

EcoRI digests
HindIII digests
SStI digests
kb
9.5-
6.4-
4.2-
2.5 × SSC. 65°C
9.5-
6.4-
4.2-

FIGURE 3

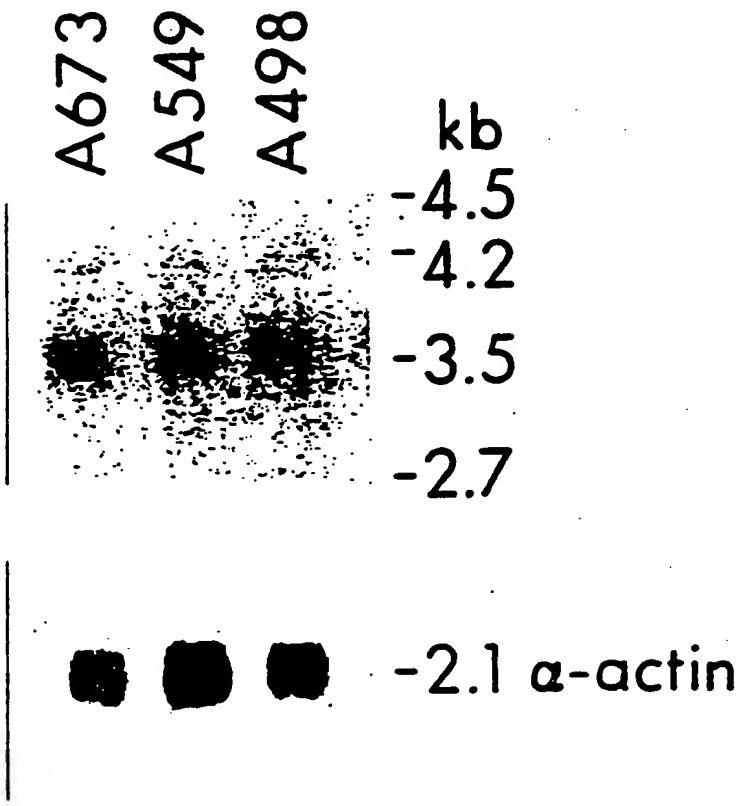


FIGURE 4

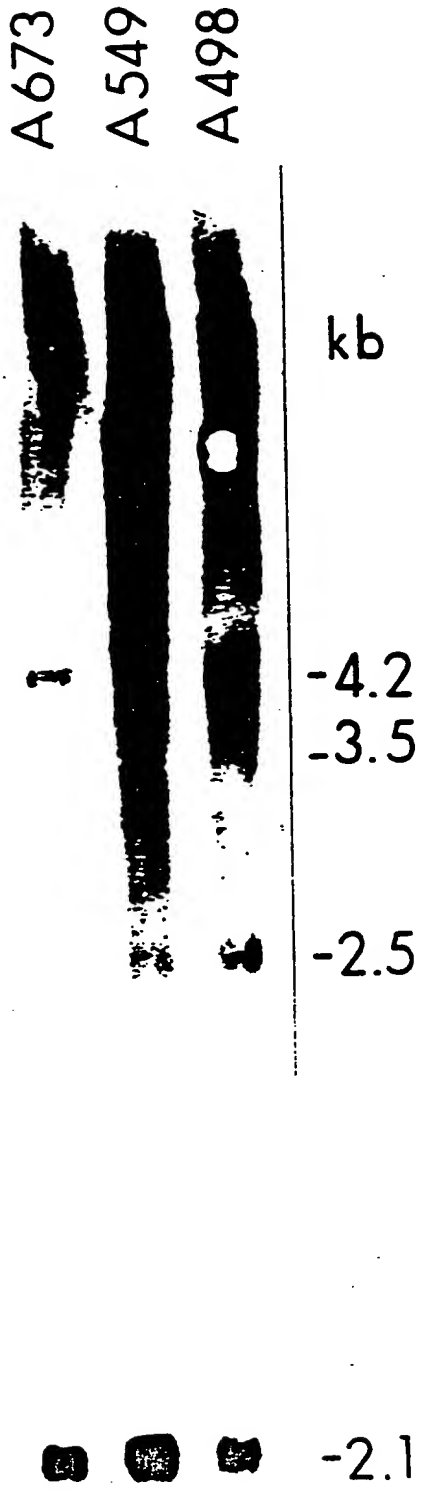


FIGURE 5

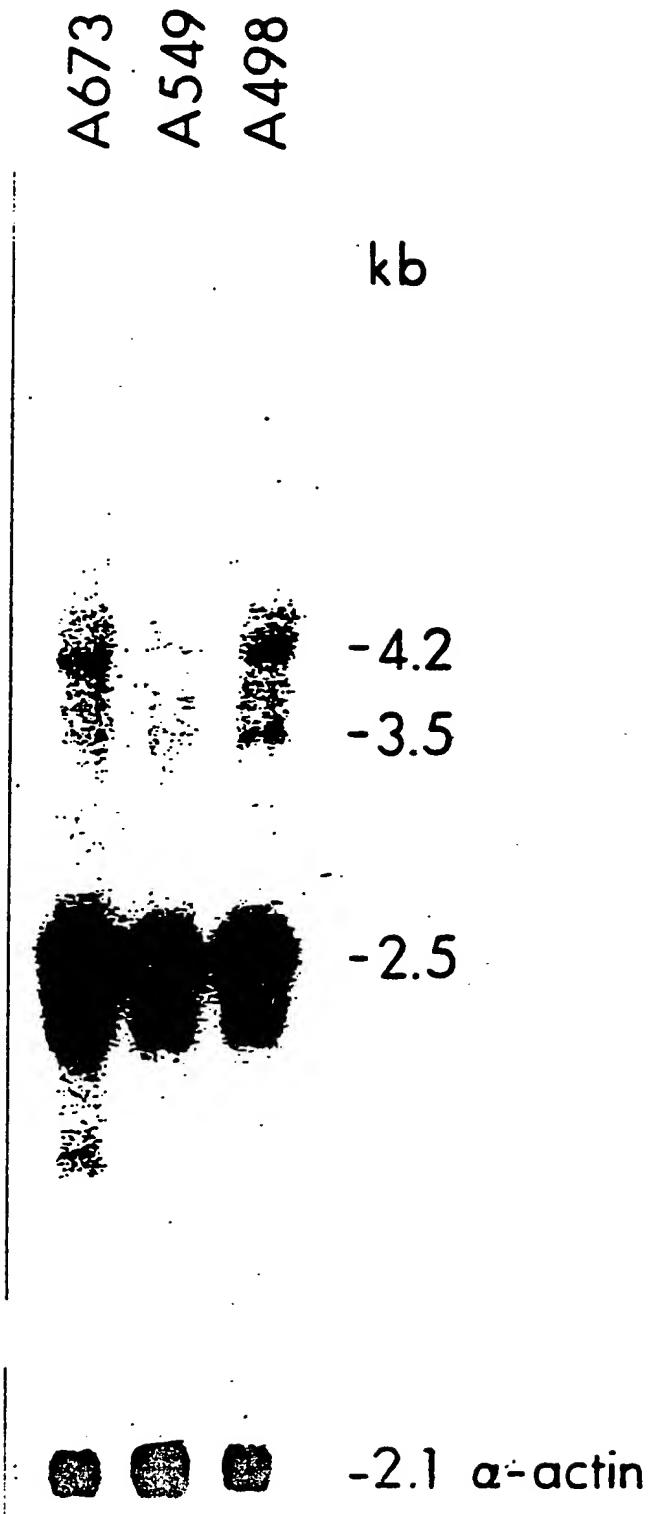


FIGURE 6

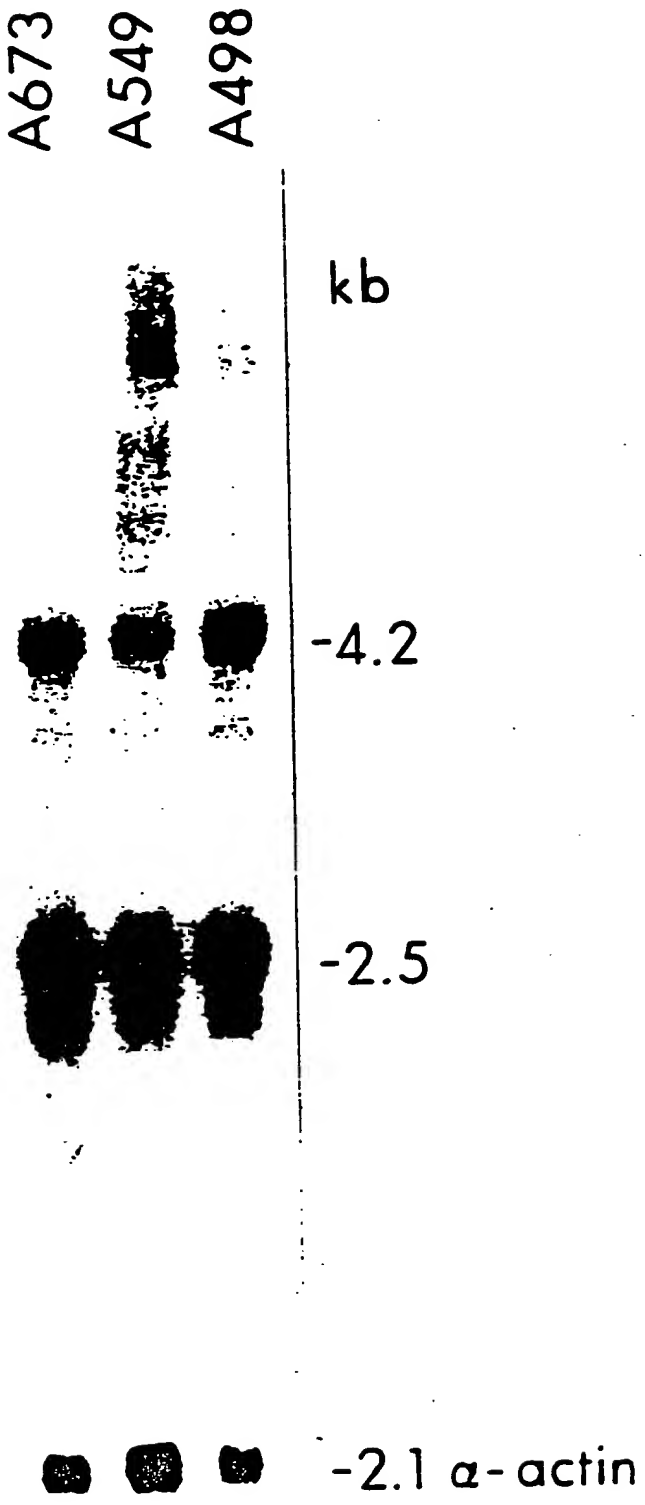


FIGURE 7

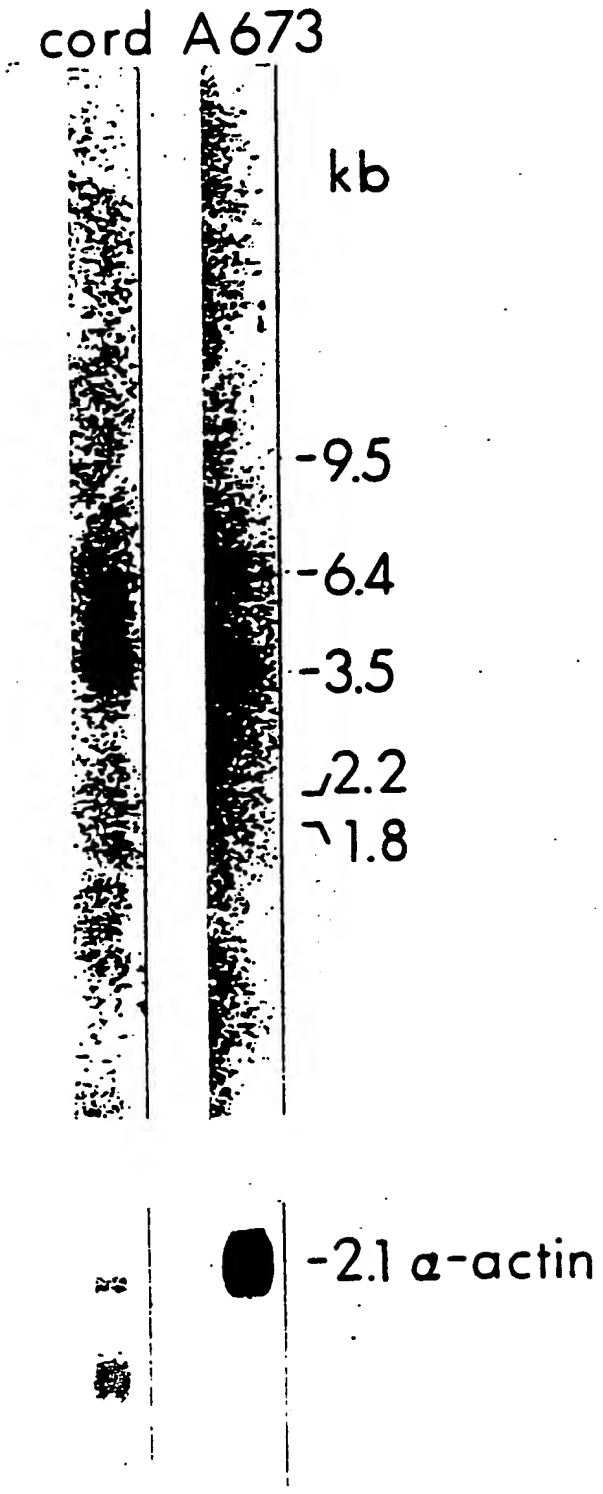
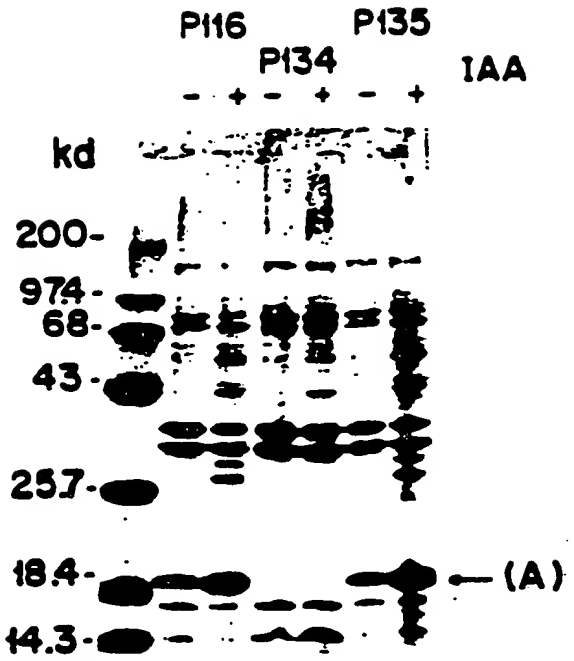


FIGURE 8



(A)= the protein having tumor growth inhibitory activity.

FIGURE 9

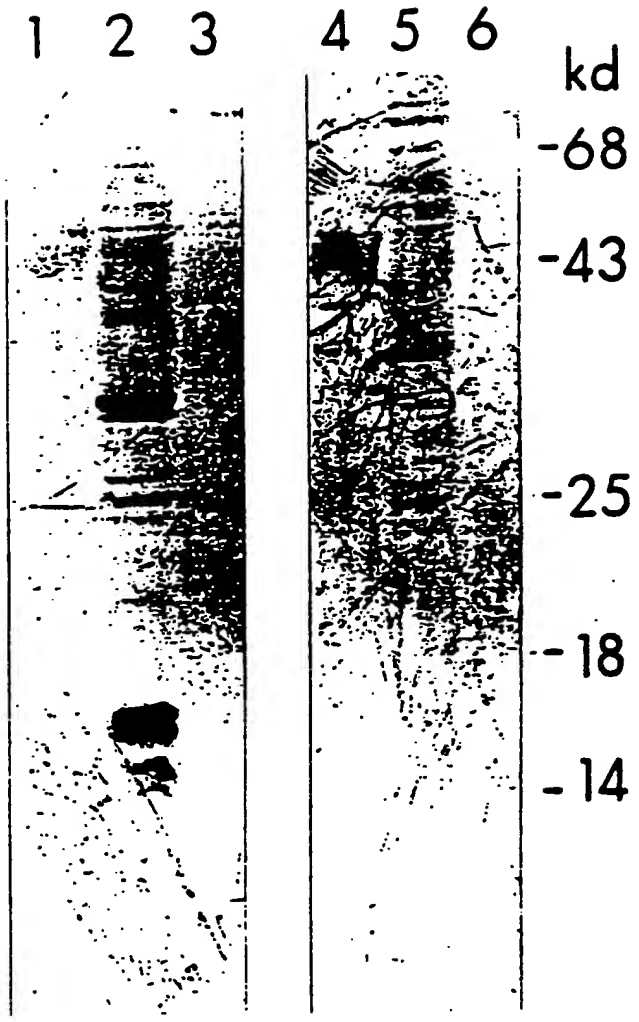


FIGURE 10

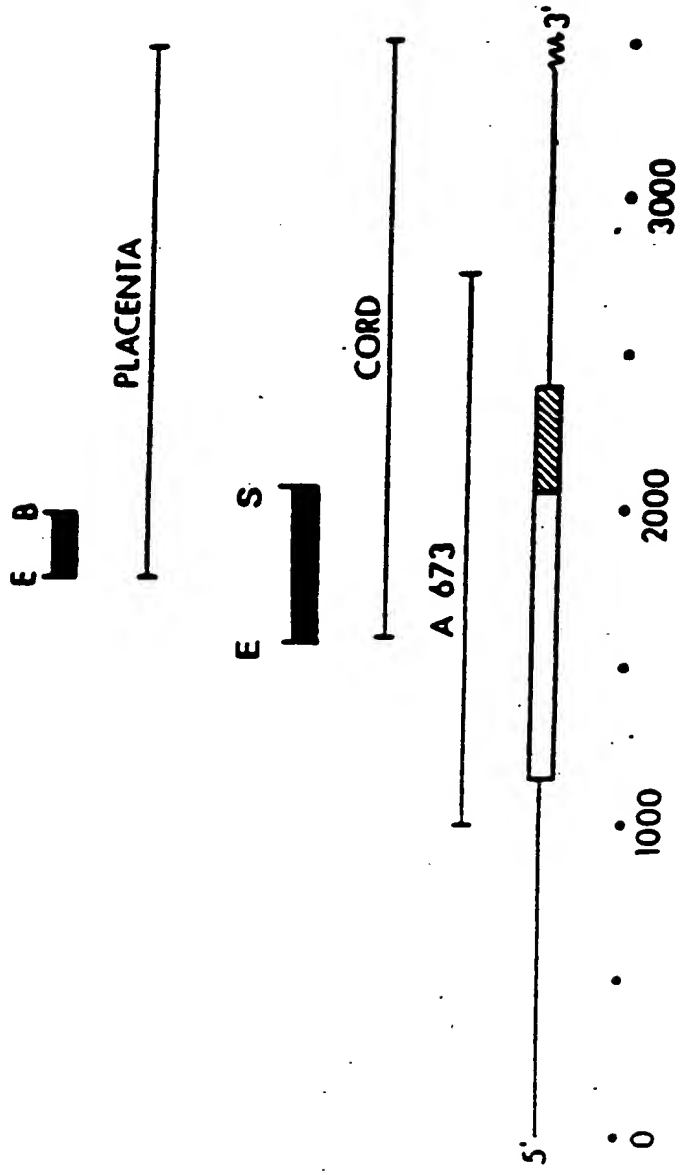


FIGURE 11

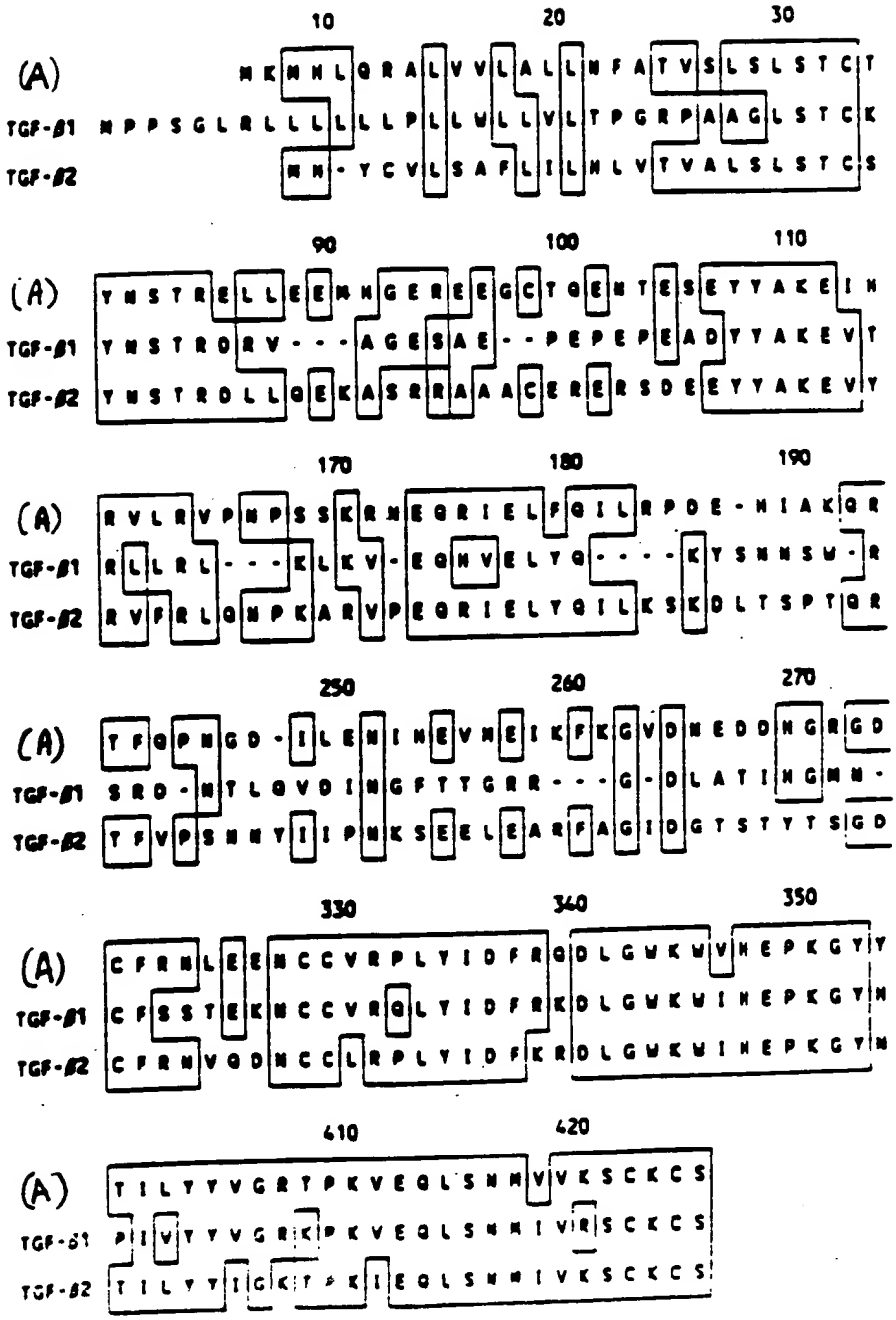

10
20
30
(A)
TGF-β1
TGF-β2
90
100
110
170
180
190
250
260
270
330
340
350
410
420

FIGURE 11

-continued-

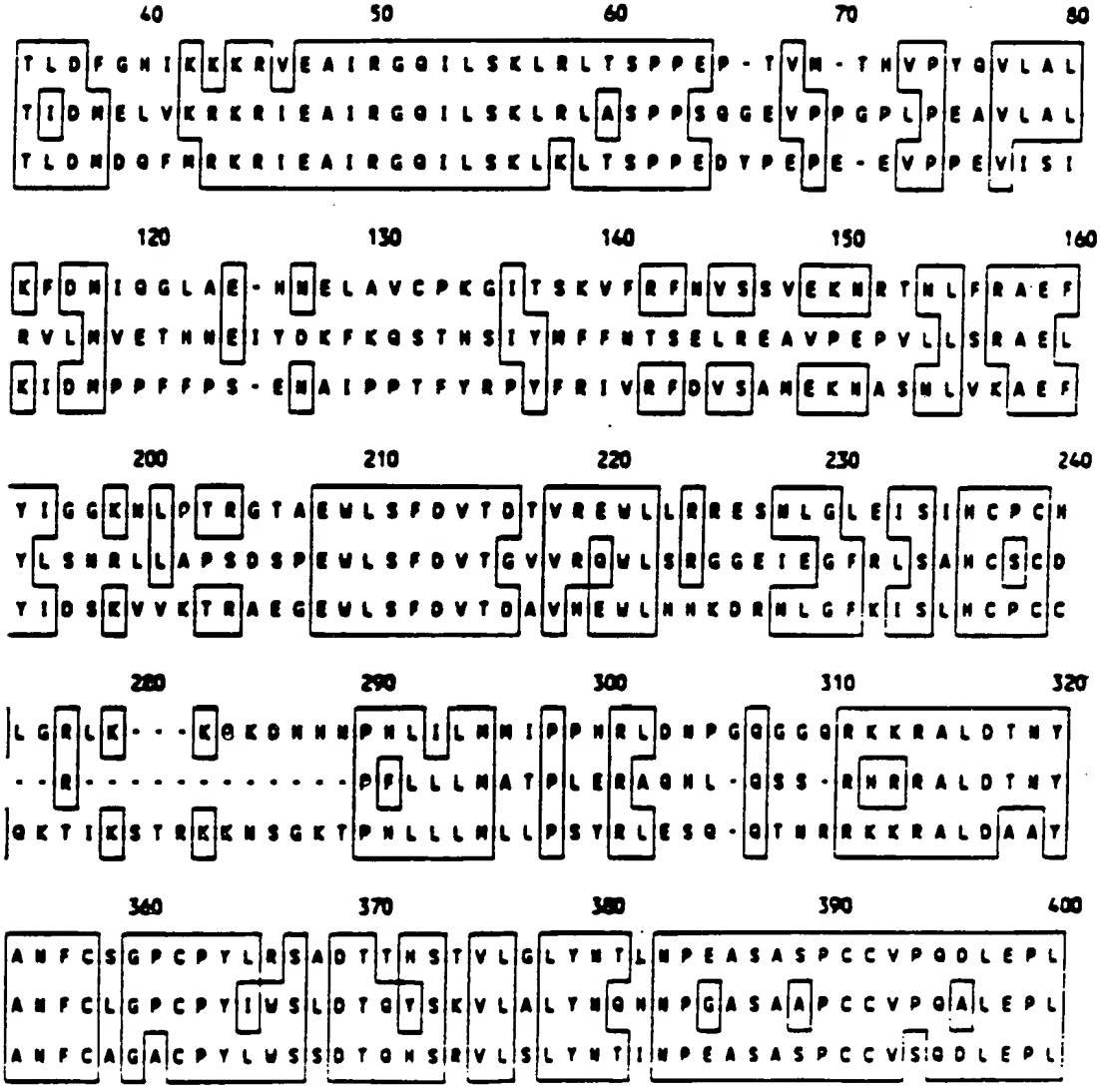


FIGURE 12

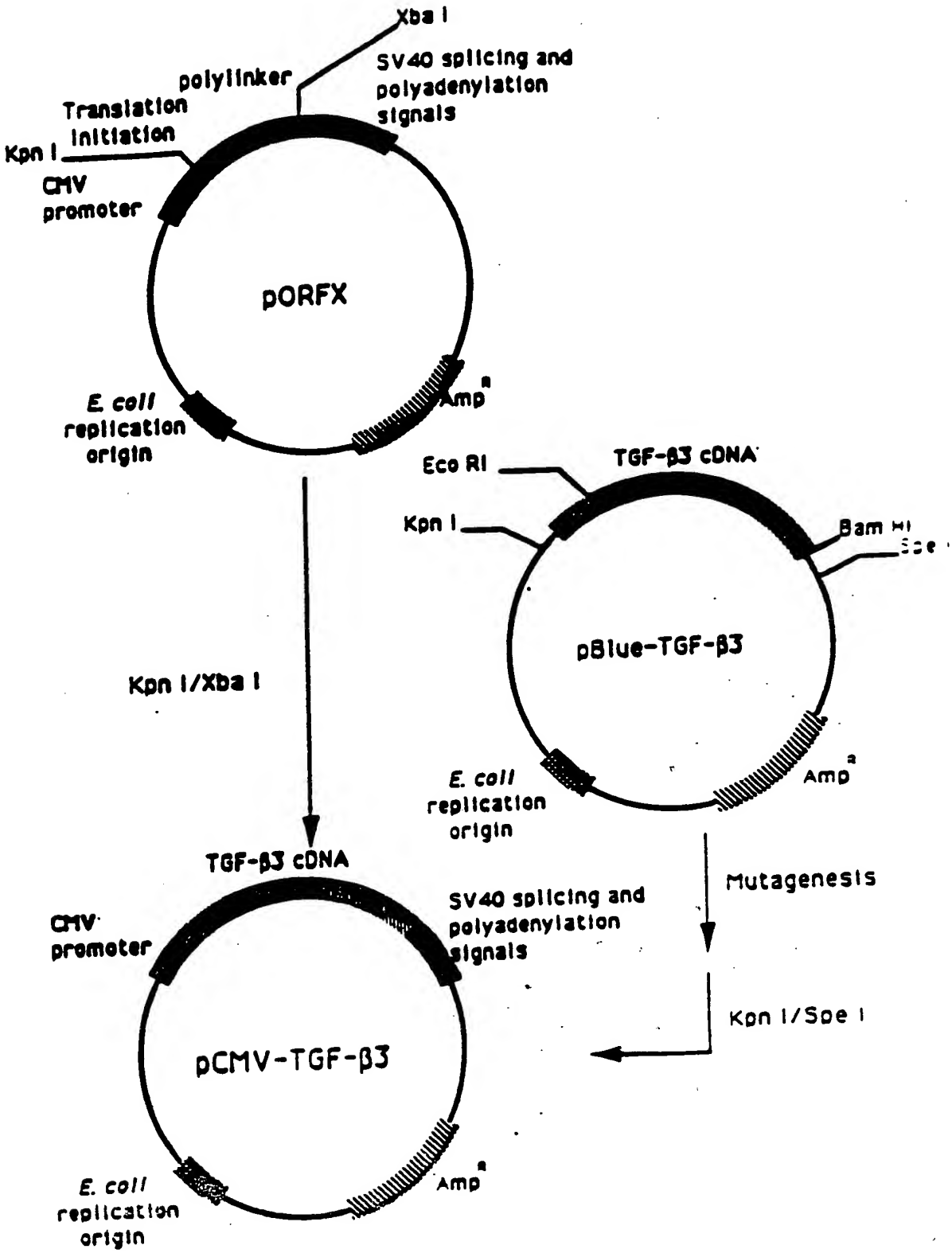

Xba I
SV40 splicing and polyadenylation signals
polylinker
Translation initiation
Kpn I
CMV promoter
pORFX
E. coli replication origin
Amp
Eco RI
TGF-β3 cDNA
Kpn I
Bam HI
Spe I
pBlue-TGF-β3
E. coli replication origin
Amp
Kpn I/Xba I
Mutagenesis
Kpn I/Spe I
TGF-β3 cDNA
CMV promoter
SV40 splicing and polyadenylation signals
pCMV-TGF-β3
E. coli replication origin
Amp

FIGURE 13

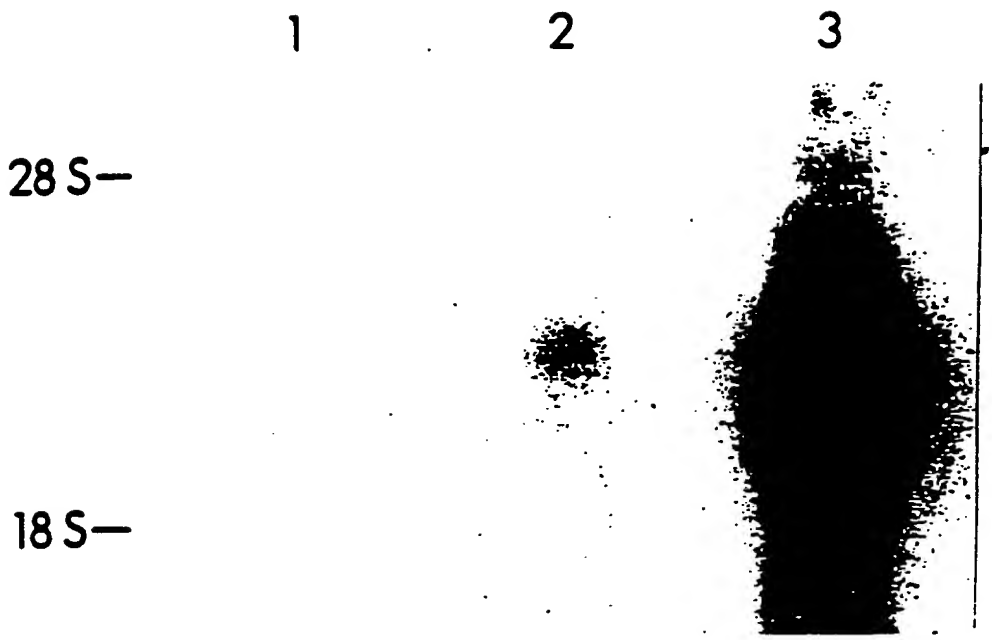
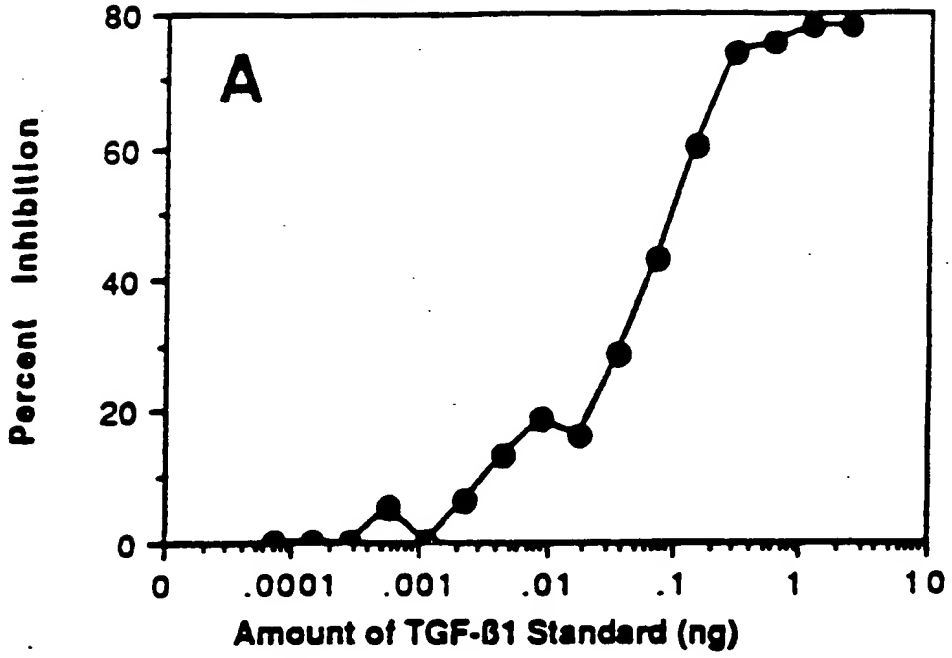


FIGURE 14


A
Percent Inhibition
80
60
40
20
0
0
.0001
.001
.01
.1
1
10
Amount of TGF-ß1 Standard (ng)

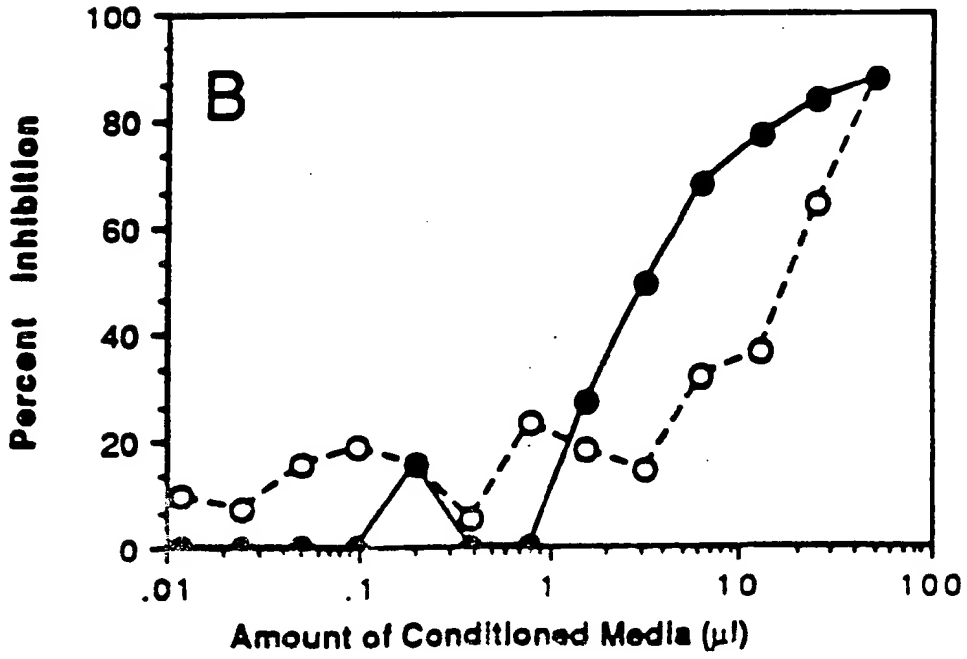

B
Percent Inhibition
100
80
60
40
20
0
.01
.1
1
10
100
Amount of Conditioned Media (µl)

FIGURE 15

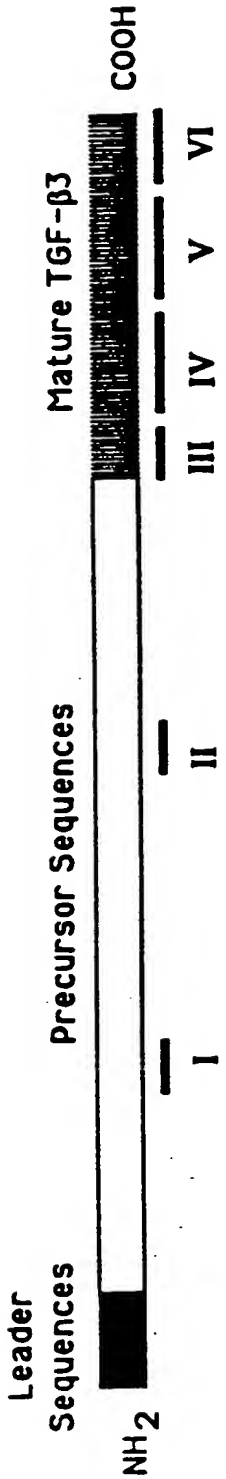

COOH
Mature TGF-β3
VI
V
IV
III
II
I
Precursor Sequences
Leader Sequences
NH2

FIGURE 16

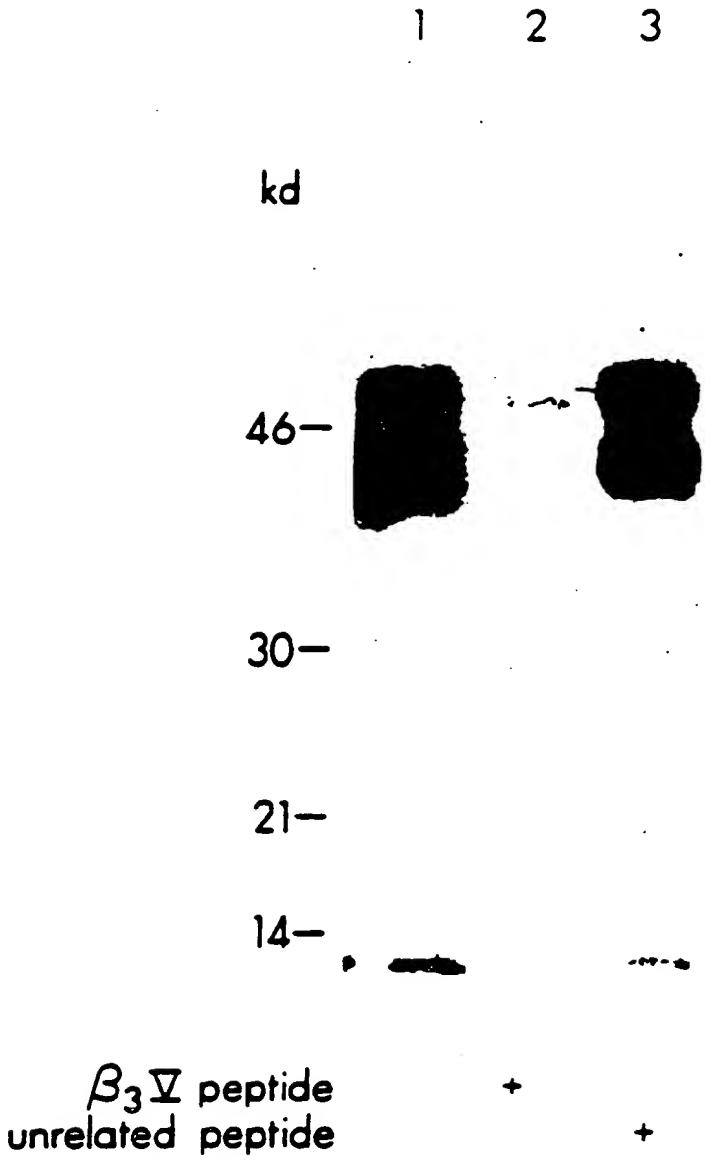


FIGURE 17

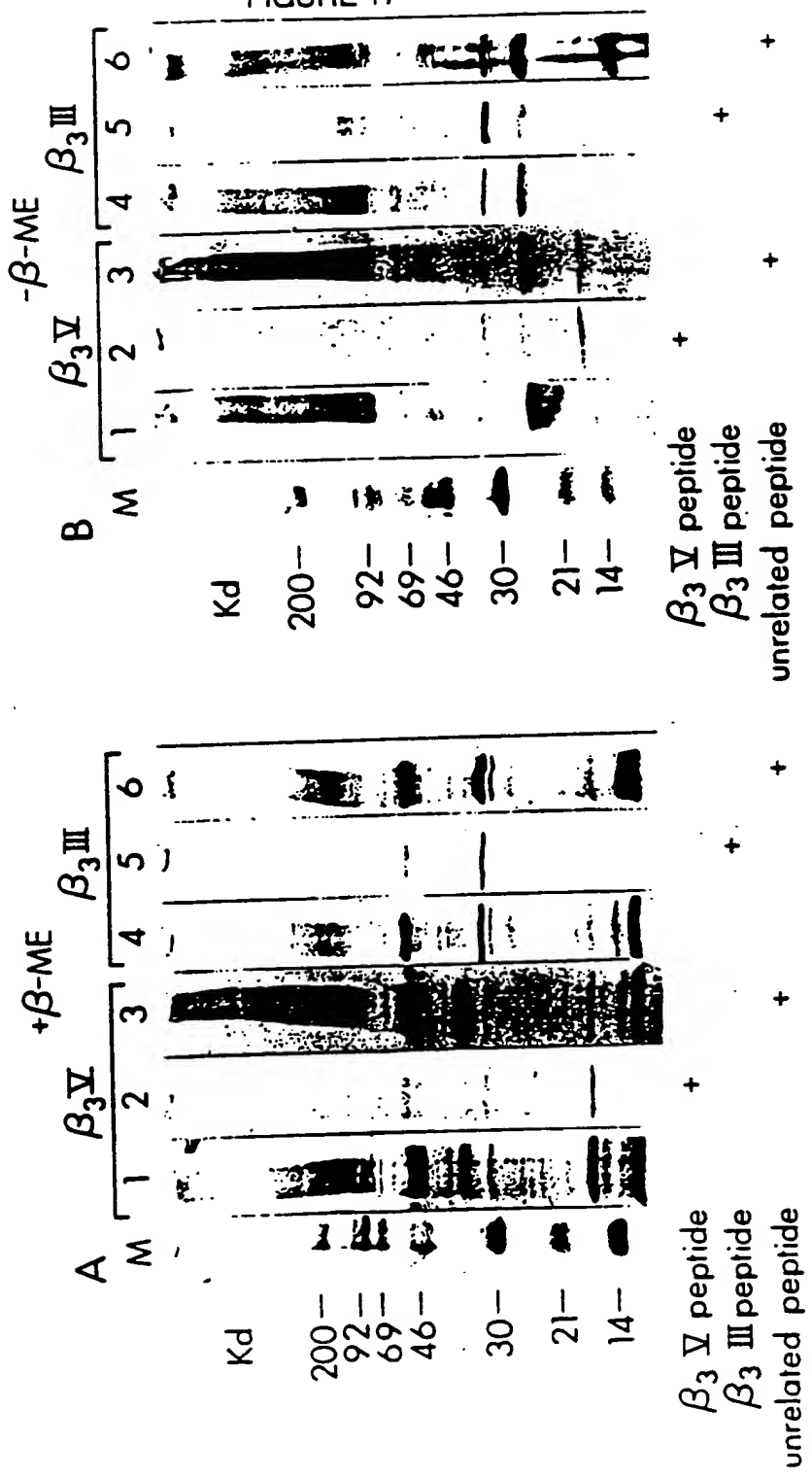

B
-β-ME
M
β3 V
β3 III
1
2
3
4
5
6
Kd
200—
92—
69—
46—
30—
21—
14—
β3 V peptide
β3 III peptide
unrelated peptide
A
+β-ME
M
β3 V
β3 III
1
2
3
4
5
6
Kd
200—
92—
69—
46—
30—
21—
14—
β3 V peptide
β3 III peptide
unrelated peptide

FIGURE 18

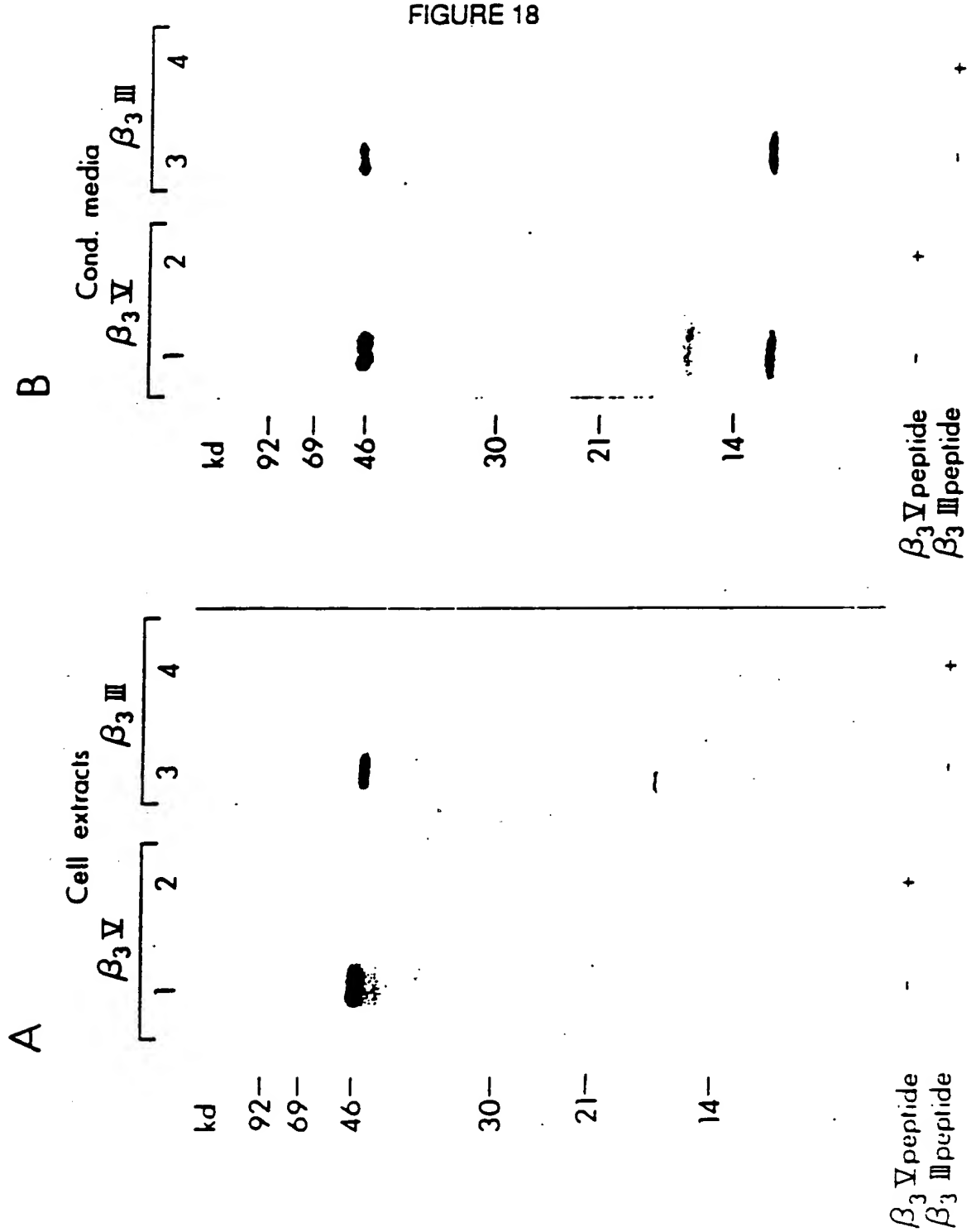

A
Cell extracts
β_3 V
1
2
β_3 III
3
4
kd
92—
69—
46—
30—
21—
14—
β_3 V peptide
β_3 III peptide
B
Cond. media
β_3 V
1
2
β_3 III
3
4
kd
92—
69—
46—
30—
21—
14—
β_3 V peptide
β_3 III peptide

FIGURE 19A

FIGURE 19B

FIGURE 19C

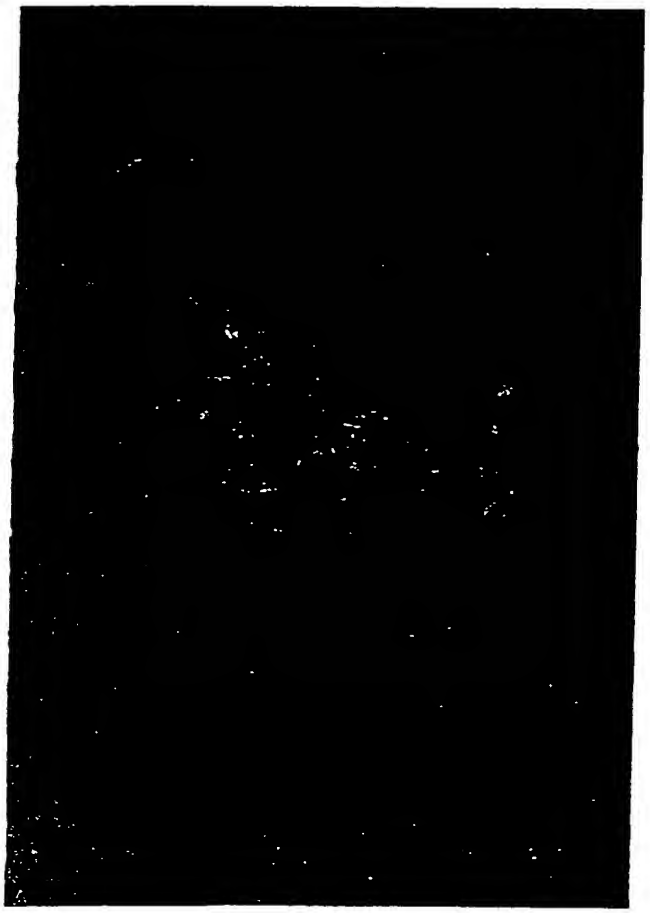


FIGURE 19D

FIGURE 20

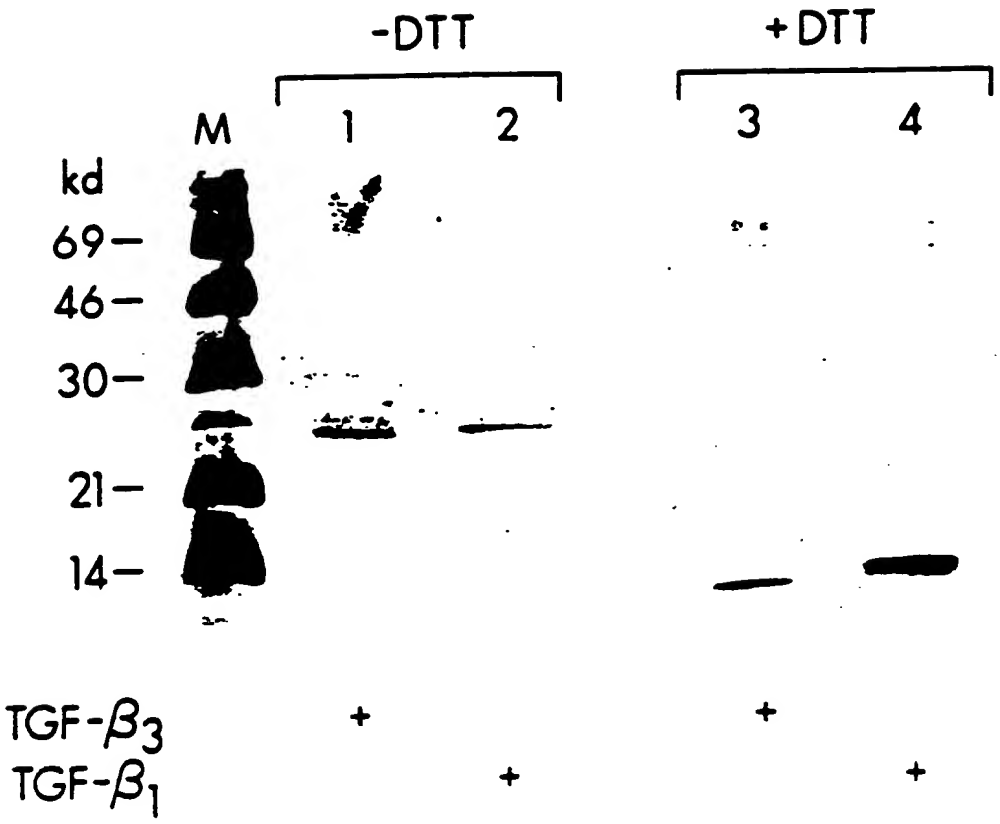


FIGURE 21A

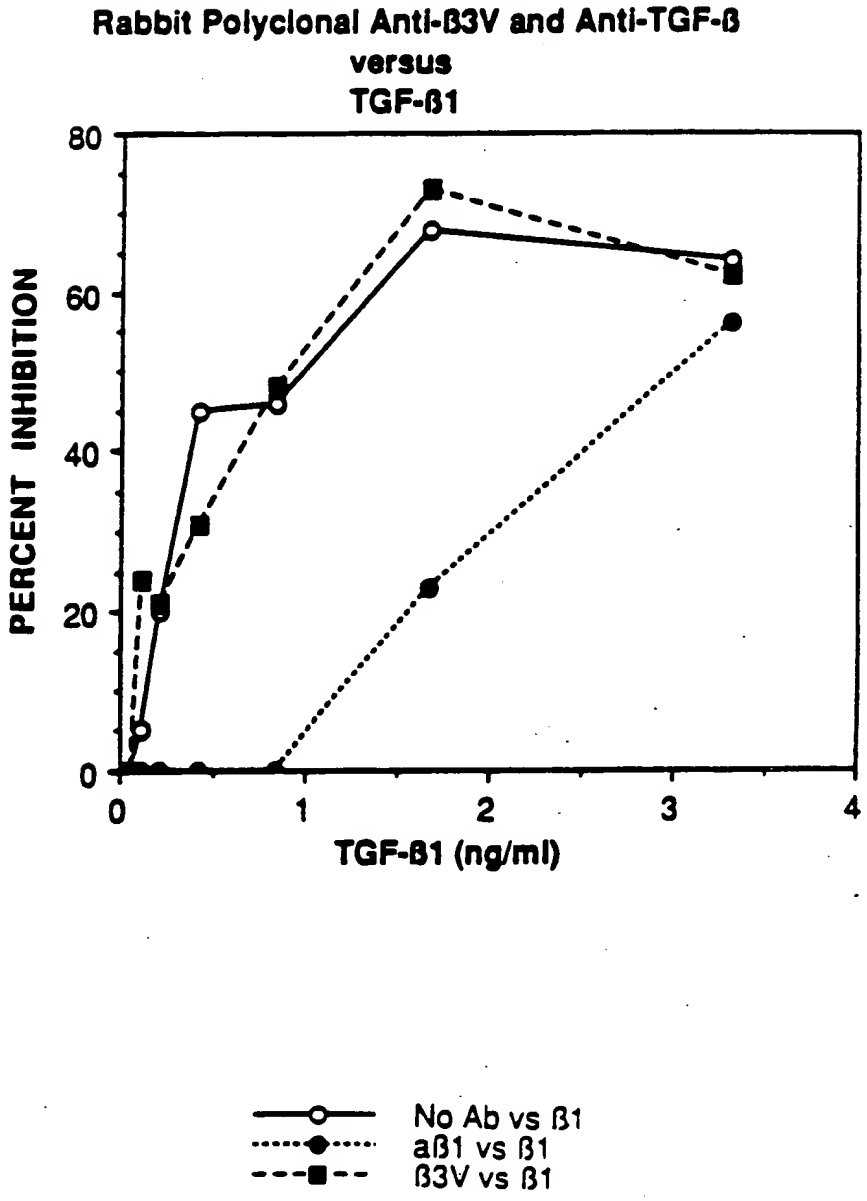

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß1
PERCENT INHIBITION
0
20
40
60
80
0
1
2
3
4
TGF-ß1 (ng/ml)
No Ab vs ß1
aß1 vs ß1
ß3V vs ß1

FIGURE 21B

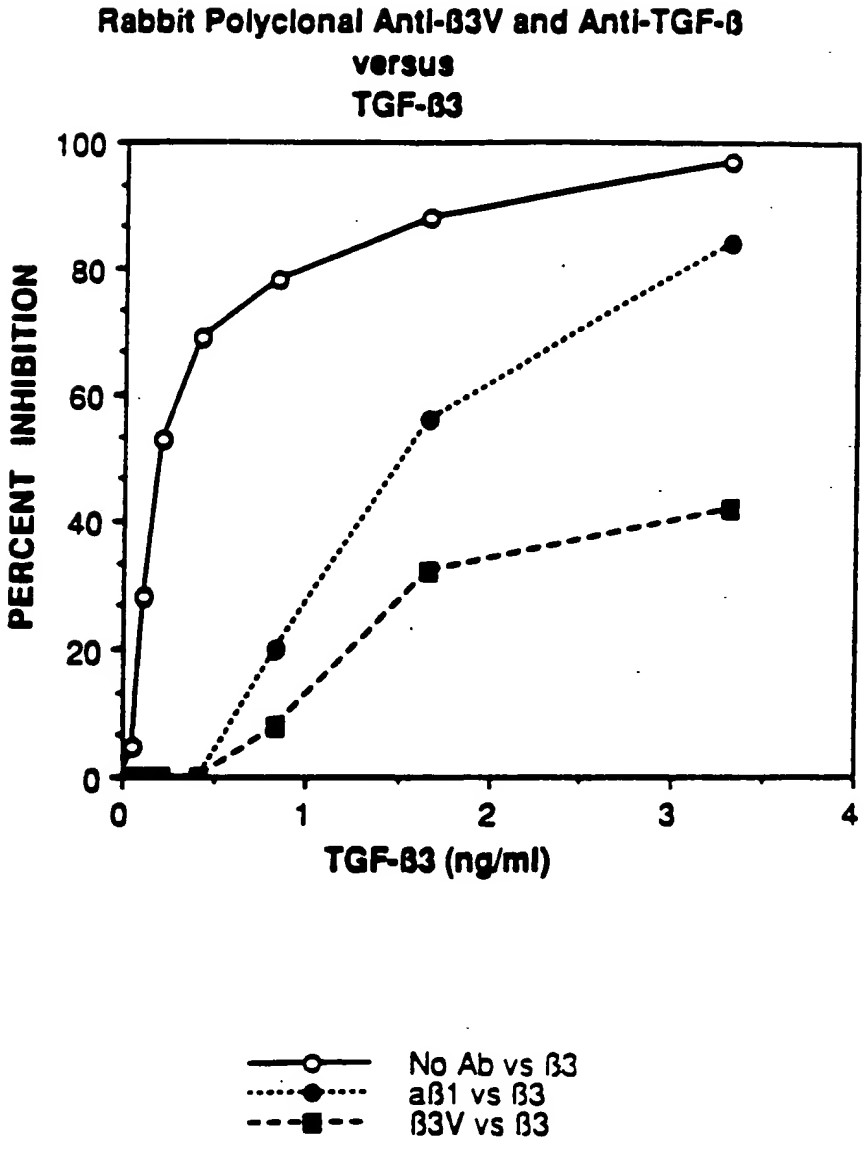

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß3
PERCENT INHIBITION
100
80
60
40
20
0
0
1
2
3
4
TGF-ß3 (ng/ml)
No Ab vs ß3
aß1 vs ß3
ß3V vs ß3

FIGURE 21C

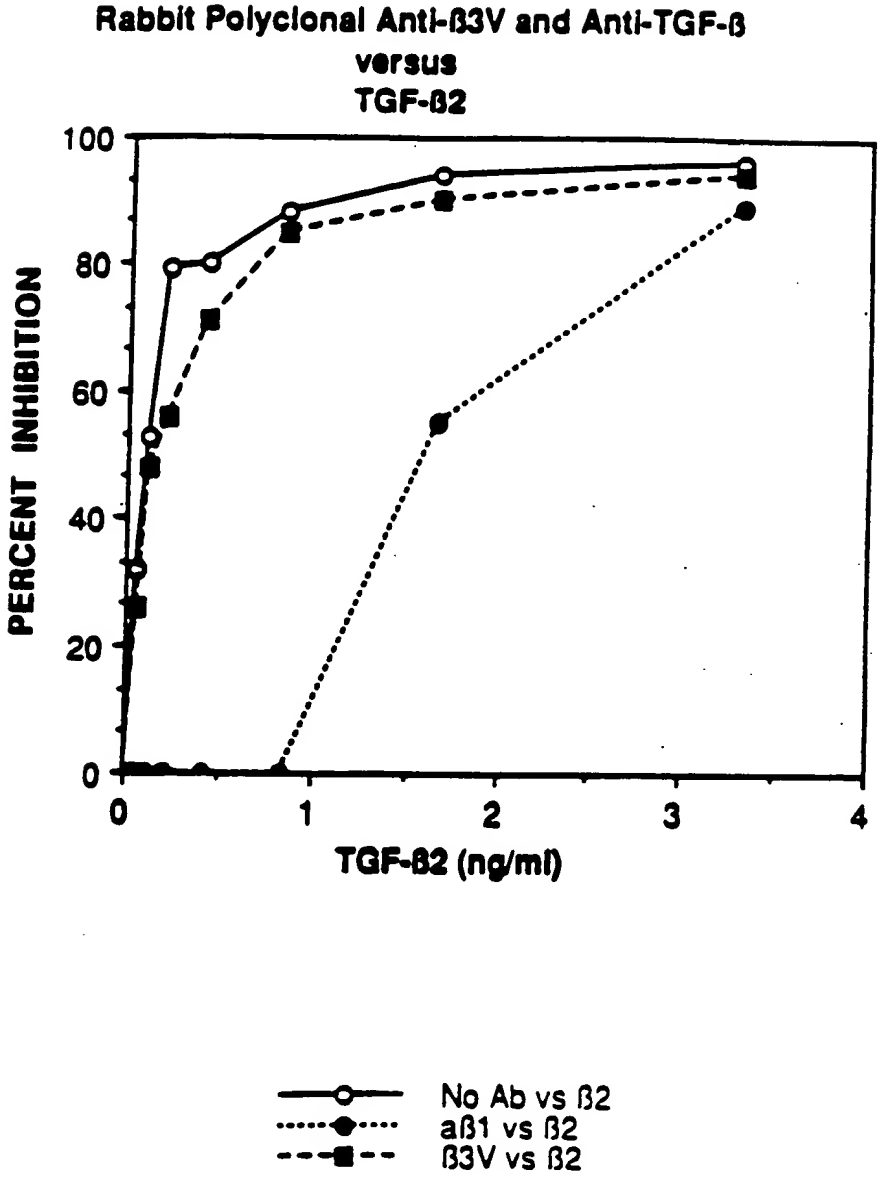
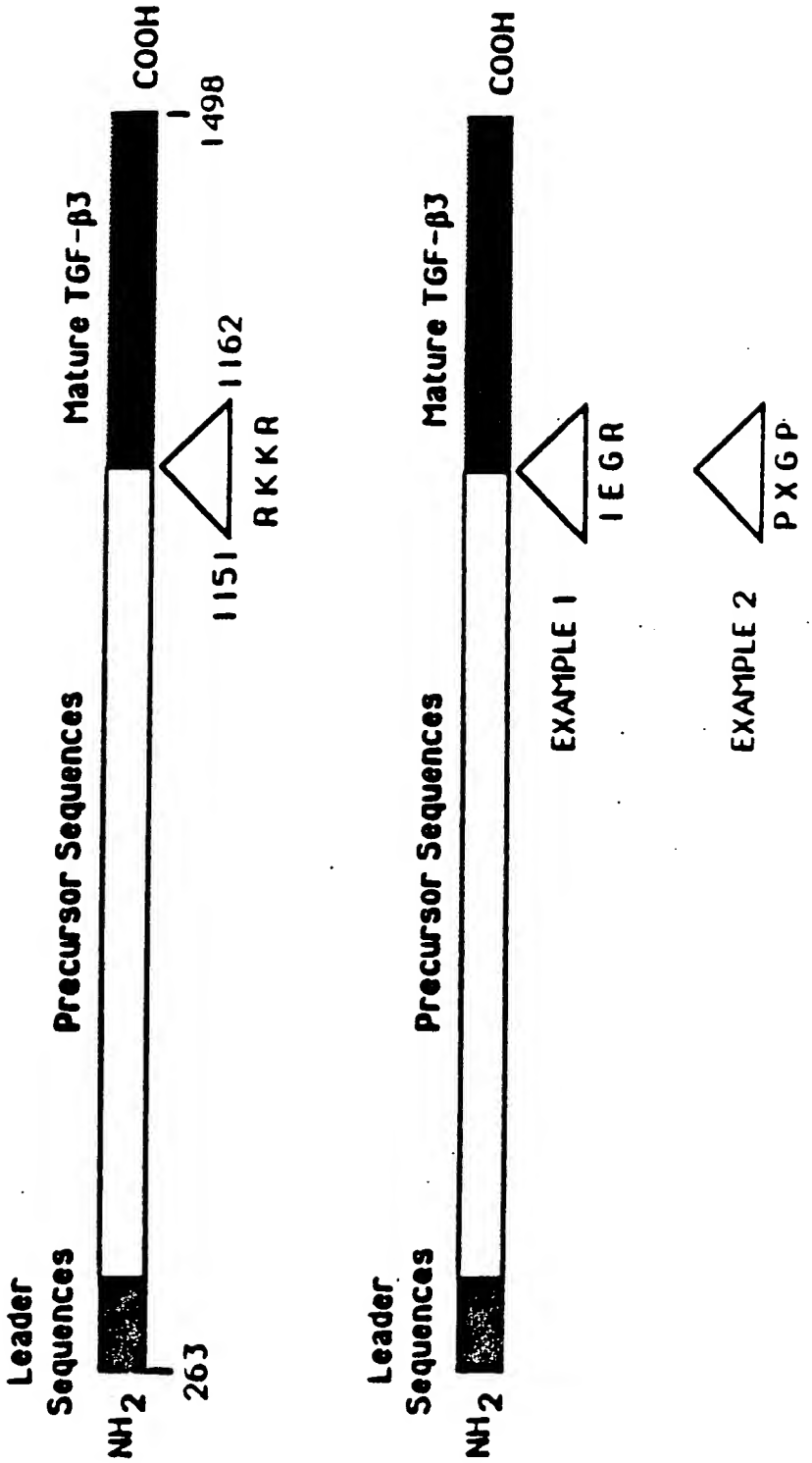

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß2
PERCENT INHIBITION
100
80
60
40
20
0
0
1
2
3
4
TGF-ß2 (ng/ml)
No Ab vs ß2
aß1 vs ß2
ß3V vs ß2

FIGURE 22


COOH
1
1498
Mature TGF-β3
1162
R K K R
1151
Precursor Sequences
Leader Sequences
NH2
263
COOH
Mature TGF-β3
I E G R
EXAMPLE 1
Precursor Sequences
Leader Sequences
NH2
EXAMPLE 2
P X G P

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04541

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 3/20, 3/24; C12P 15/00
US. CL.: 530/413, 420; 435/69.4

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/412, 413, 418, 419, 420; 435/68.1, 69.1, 69.4, 70.1, 172.1, 172.3

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (Files 5,73,155); USPTO Automated Patent System (File USPAT, 1971-1991).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,708,948 (IWATA et al.) 24 November 1987, See entire document.	1-37
Y	US,A, 4,886,747 (DERYNCK et al.) 12 DECEMBER 1989. See entire document, particulary Fig. 4a-4c and Example 6, column 23.	1-37
Y	The EMBO JOURNAL, Vol. 7, No. 12, issued 1988, Derynck et al., "A New Type of Transforming Growth Factor-B, TGF-B3," pages 3737-3743, See entire document, particularly Fig. 1 Sequence.	1-37
Y	US,A, 4,931,548 (Lucas et al) 05 June 1990, see entire document.	1-37

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 October 1991	13 NOV 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Robert D. Budens

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-4, drawn to methods of purifying TGF-B3 antibodies.

II. Claims 5-21 and 34-37, drawn to methods of producing TGF-B3 precursors.

III. Claims 22-23, 28-33 and 36-37 drawn to methods of producing mutant. TGF-B3.

IV. Claims 24-27, drawn to processes for producing TGF-B3 using precipitation

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED T BE RELEVANT (C NTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Vol. 85, issued July 1988, Ten Dijke et al., "Identification of Another Member of the Transforming Growth Facotr Type B Gene Family," pages 4715-4719, See entire document, especially Figures 2 and 3.	1-37
X, P Y	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, Vol. 593, issued 26 JUNE 1990, Ten Dijke et al., "Molecular Characterization of Transforming Growth Factor Type B3." pages 26-42, See entire document.	1-4, 5-37
Y,P	MOLECULAR AND CELLULAR BIOLOGY, Vol. 10, NO. 9, issued SEPTEMBER 1990, Ten Dijke et al. "Recombinant Transforming Growth Factor Type Type B3: Biological Activities and Receptor-Binding Properties in Isolated Bone Cells," pages 4473-4479, see entire document.	1-37
A	CANCER research, Vol. 45, issued JUNE 1985, IWATA ET AL., "Isolation of Tumor Cell Growth Inhibiting Factors from a Human Rhabodomyo-sarcoma Cell Line, " pages 2689-2694.	1-37
A	CANCER RESEARCH, Vol. 45 issued JUNE 1985, Fryling et al., "Two Distinct Tumor Cell Growth inhiniting Factors form a Human Rhabdomyosarcoma Cell Line," pages 2695-2699.	1-37